-continued

	3 ml

***GM stimulators include but are not limited to various factors which can be tested for colony stimulation, as described for the CFU-GM assay. The volume of the GM stimulator, and thus of the IMDM, may vary with the type of stimulator used (e.g., mouse = PWMSCM; Human = 5637 CM or PHALCM). Also note that IMDM is strictly a compensation for the remaining volume of 3 l.

3. Mix suspension thoroughly by vortexing and inversion of tubes.
4. After allowing bubbles to rise from the mixture, place 1 ml mixture in each of two 10×35 mm culture plates containing erythropoietin, hemin, and colony stimulating factors, if so desired. Rotate the plates so that the mixture coats the surface of the plates.
5. Place these 2 plates in a large 15×100 mm petri dish along with a 10×35 mm humidifying dish containing about 1 ml of H_2O. Replace the lid of the large dish.
6. Place the petri dish in an appropriate incubator for 14 days. Conditions of incubation are the same as described for the CFU-GM assay of Section 6.6.1.
7. Remove plates from the incubator and score by observation of colonies under an inverted or stereoscopic microscope.

In some cultures, the GM stimulator/burst-promoting activity (e.g., medium conditioned by 5637 cells or PHALCM) can be omitted; under these conditions, the assay detects a more mature population of BFU-E (BFU-E-2) cells and few or no CFU-GEMM cells.

6.6.2.1. Preparation of 2.1% Methyl Cellulose

The 2.1% methylcellulose, for use in the BFU-E/CFU-GEMM assay, was prepared as follows:

Stock solution:	
2.1% Methocel (Dow Chemical Co.)	21 grams
Boiling water	500 ml
2x IMDM	500 ml

Procedure:

The gram weight of methyl cellulose is put into a sterile 3 liter Erlenmeyer flask (having a sterile stopper) containing a sterile magnetic flea on a large magnetic stirrer. To prevent as little frothing as possible, stirring is initiated while 500 ml of sterile boiling distilled H_2O is gently poured down the sides of the flask. Stirring continues at room temperature until the flask gradually cools (this may take an hour). When the flask is no longer hot to the touch, 500 ml of 2×IMDM, which had been allowed to come to room temperature, is added to the flask without frothing. The flask is stoppered and transferred to the cold room (4° C.) where stirring continues for 48 hours. The solution is then sterilely aliquoted into sterile 100 ml bottles. The bottles are stored frozen for up to 6 months (protected from light).

6.6.2.2. Preparation of Hemin

The hemin, for use in the BFU-E/CFU-GEMM assay, was prepared as follows:

260 mg	Hemin (Eastman Kodak #2203)
4 ml	0.5M NaOH
5 ml	Tris buffer, 1 M, pH 7.8 (approximately

-continued

	9.5 parts acid to 3 parts base)

Bring to 100 ml with double-distilled H_2O.

1. Dissolve hemin in NaOH completely before adding Tris buffer and H_2O.
2. After adjusting the volume to 100 ml, filter-sterilize by passage through an 0.45 um filter, and store in 2–3 ml aliquots at −20° C.

6.6.2.3. Preparation of Iscove's Modified Dulbecco's Medium

1×Iscove's Modified Dulbecco's Medium (IMDM), for use in the BFU-E/CFU-GEMM assay, was prepared as follows:

1. Measure out 5% less water (deionized, distilled) than desired total volume of medium, using a mixing container that is as close to the final volume as possible.
2. Add powder medium (Gibco Laboratories, Formula No. 78-5220), to water with gentle stirring at room temperature (do not heat water).
3. Rinse out the inside of the package, to remove all traces of the powder.
4. Add 3.024 grams of $NaHCO_3$ per liter of medium.
5. Dilute to the desired volume with water. Stir until dissolved.
6. Do not adjust pH. Keep container closed until medium is filtered.
7. Sterilize immediately by Nalgene filtration.

To prepare 1 liter of 2×liquid medium, follow the above procedure, except use 2 envelopes of powder instead of one, and 6.048 gm $NaHCO_3$.

6.6.3. Stem Cell Colony Forming Unit Assay

The assay used for stem cell (S-cell) quantitation does not directly assay self-renewal, but instead assays for the ability to generate secondary multilineage colonies on replating. This assay is done essentially the same as the BFU-E/CFU-GEMM assays, except that cultures are scored after 21–28 days of incubation rather than after 14 days (for BFU-E and CFU-GEMM). The drug 4-hydroperoxycyclo-phosphamide (4HC) appears to spare immature progenitors at the expense of mature progenitors, and may be useful for pretreating cells before assay. Factors which can be tested for increasing the self-renewal ability of S-cells in vitro (thus increasing assay efficiency) include but are not limited to hemin, oxygen tension (Smith, S. and Broxmeyer, H. E., 1986, Brit. J. Haematol. 63:29–34), superoxide dismutase, glucose oxidase, IL-3, GM-CSF, G-CSF, M-CSF, erythropoietin, IL-1, IL-4, etc.

6.6.4. Assay of the Proliferative Status of Stem and Progenitor Cells

The proliferative status of stem and progenitor cells can be measured by a high specific activity tritiated thymidine (^{3}HTdr) kill (or suicide) technique, carried out as follows:

1. In two small 12×75 mm polystyrene tubes, place the appropriate volume of stock cell suspension containing 2–3 times the number of cells required for plating. (For bone marrow, 2–3×10^6 cells and for spleen, 15–20×10^6 cells. For cord blood: 2–3×10^6 (approx.) cells.) Label them a and b.
2. Pellet the cells by centrifugation at 200–400×g at 4° C. for 10 minutes.
3. Carefully remove and discard the supernatant.
4. Add 0.5 ml of McCoy's 5A medium supplemented as prescribed in Section 6.6.1.1, supra, and with FCS at 10% V/V.

5. To tube b, add 50 uCi of ^{3}HTdr (New England Nuclear, #NET-027×Thymidine, [methyl-^{3}H]-20.0 Ci/mmol; 5.0 mCi/5.0 ml H_2O). As a control, to tube a, add 50 ul of McCoy's 5A medium.
6. Place cap back on tubes and gently vortex in order to resuspend cells.
7. Place the tubes in a tray also containing H_2O, in an incubator with an atmosphere of 5% CO_2, and a temperature of 37° C., for 20 minutes.

TABLE V

RECOVERY OF CORD BLOOD HEMATOPOIETIC PROGENITOR CELLS AFTER FREEZE-THAWING

			Total Number of Hematopoietic Progenitor Cells per 4 × 10^6 Frozen Cells				
Sample	Pre-/Post-Freeze-Storage	No. Viable Cells × 10^{-6}	CFU-GM Day 7	CFU-GM Day 14	BFU-E-2	BFU-E-1	CFU-GEMM
CB-1	Pre	3.7	1332	5254	3848	2146	3108
	Post	2.5	284	2162	1680	1652	1205
% Survival after Thaw		67.6	21.3	41.1	43.7	77.0	38.8
CB-2	Pre	3.8	3268	6004	4028	4104	4864
	Post	1.4	1902	2688	2361	1911	990
% Survival after Thaw		36.8	58.2	44.7	58.6	46.6	20.4
CB-3	Pre	3.8	912	4408	2964	2356	2660
	Post	1.6	746	2782	1702	1826	742
% Survival after Thaw		42.1	81.8	63.1	57.4	77.5	27.9
CB-10	Pre	3.9	1014	7800	3354	3120	2964
	Post	1.2	1300	3175	1526	829	794
% Survival after Thaw		30.8	128.2	40.7	45.4	26.6	26.8
CB-14	Pre	3.8	1900	3724	4484	3952	3344
	Post	1.1	1034	2672	1194	1240	892
% Survival after Thaw		28.9	54.4	71.8	26.6	31.4	26.7
CB-15	Pre	3.6	720	2160	3096	2448	1512
	Post	1.7	426	2424	1170	1062	740
% Survival after Thaw		47.2	59.2	112.2	37.8	43.4	48.9
CB-16	Pre	3.7	518	1110	592	1036	740
	Post	0.8	112	548	190	280	143
% Survival after Thaw		21.6	21.6	49.4	67.9	27.0	19.3
CB-17	Pre	3.7	0	0	592	1332	592
	Post	0.5	190	550	170	360	210
% Survival after Thaw		13.5	100	100	28.7	27.0	35.5
Average % Survival after Thaw:		36.1	65.6	65.4	45.8	44.6	30.5
Range ( )		(13.5–67.6)	(21.3–128.2)	(40.7–112.2)	(28.7–67.9)	(26.6–77.5)	(19.3–48.9)

8. Add 0.5 ml (2.5 mg) of ice cold (4° C.) "cold" (nonradioactive) thymidine (Sigma #T-9250) at 5 mg/ml to each tube, and vortex lightly. Add an additional 2 ml of ice cold McCoy's 5A medium to each tube.
9. Pellet cells by centrifugation at 200–400×g at 4° C. for 10 minutes.
10. Aspirate the supernatant into an appropriate container (one used for radioactive disposal), and resuspend the cells with 2 ml cold medium. Repeat step #10.
11. Aspirate the supernatant into an appropriate container. Resuspend with McCoy's 5A containing 10% FCS to a volume where the cell concentration is at least 10 fold greater than the plating concentration.
12. Keep cells on ice until ready to plate.
13. Plate and carry out colony forming assays as described supra in sections 6.6.1 through 6.6.3.

6.7. Recovery After Freeze-thawing of Human Hematopoietic Progenitor Cells Derived from Cord Blood

The results of progenitor cell assays after freeze-thawing were compared to results of the same assays obtained before freeze-thawing, in order to assess the recovery of hematopoietic progenitor cells from human cord blood after the freeze-thawing process. Eight cord blood samples, obtained as described in Section 6.1, supra, and separated by use of Ficoll-Hypaque, were analyzed. The results are shown in Table V.

As shown in Table V, the average % survival after freeze-thawing was 36.1, 65.6, 65.4, 45.8, 44.6, and 30.5, respectively, for nucleated cells, day 7 CFU-GM, day 14 CFU-GM, BFU-E-2, BFU-E-1, and CFU-GEMM. There was a range of variability in recovery rates.

It should be noted that the data presented in Table V reflects cell losses incurred during Ficoll-Hypaque separations and procedures for DMSO removal, two steps which are omitted in a preferred embodiment of the invention (NB: DMSO should be removed before colony assays if such are desired to be carried out).

6.8. Calculations of the Reconstituting Potential of Cord Blood

The following discussion demonstrates that individual collections of cord blood (such as described in Section 6.1) contains sufficient hematopoietic stem and progenitor cells to repopulate the hematopoietic system of an individual.

A survey of published reports indicates that the number of CFU-GM infused for autologous bone marrow reconstitution in human patients, can be relied on as an indicator of the potential for successful hematopoietic reconstitution (Spitzer, G., et al., 1980, Blood 55(2): 317–323; Douay et al., 1986, Exp. Hematol. 14:358–365). By standardizing

published data by patient weight, and assuming a patient weight of 150 pounds (67.5 kilograms), the calculated number of CFU-GM needed for successful hematopoietic reconstitution using autologous bone marrow cells ranges from $2–425\times10^4$, with faster recovery noted using greater than 10×10^4 CFU-GM.

The data presented in Table III, supra, for 81 cord blood collections, analyzed for day 14 CFU-GM count, shows a range of $0–109\times10^4$ CFU-GM per Ficoll-Hypaque-separated individual blood collections. Seventy samples contained greater than or equal to 2×10^4 CFU-GM, while thirty samples contained greater than or equal to 10×10^4 CFU-GM. It should be emphasized that this data is derived from Ficoll-Hypaque-separated cells obtained by either gravity drainage from the cord or needle aspiration from the delivered placenta. In a preferred embodiment of the invention, where whole blood is both frozen and infused for therapeutic use, losses due to Ficoll-Hypaque separation can be avoided (see Table IV and Section 6.3.1 infra for data on cell losses incurred during Ficoll-Hypaque separations). In addition, as mentioned in Section 6.1, supra, in recent blood collections, we have been able to obtain volumes approximately twice as large as shown in FIG. 2 or described in Table III, by using needle aspirations from the delivered placenta at the root of the placenta and in the distended surface veins, in combination with cord drainage. Furthermore, an adjustment of the collection protocol to provide for immediate cord clamping upon delivery should result in receipt of greater blood collection volumes (See Yao, A. C., et al., Oct. 25, 1969, Lancet:871–873, wherein collected neonatal blood, obtained by drainage from the umbilical cord and from the delivered placenta, averaged 126.6 ml volume when the umbilical cord was clamped in less than 5 seconds after birth). Thus, although an analysis of the data of Table III should be adjusted for expected losses during freeze-thawing (which losses, however, should not exceed 35%), there should be sufficient cord stem and progenitor cells per collection sample to successfully effect hematopoietic reconstitution.

Furthermore, the reconstituting capacity of cord blood hematopoietic cells may be higher than that of an equal number of bone marrow cells. Colonies derived from cord blood cells are usually larger in size than those derived from adult bone marrow.

6.9. In Vitro Culture Conditions for Hematopoietic Stem and Progenitor Cells

Culture conditions for the growth in vitro of hematopoietic progenitor cells from human cord blood have been described in Smith, S. and Broxmeyer, H. E., 1986, British Journal of Hematology, Vol. 63, pp. 29–34, which is incorporated by reference herein in its entirety. Culture media was composed of the following ingredients:

- RPMI 1640 media (Gibco Laboratories, Grand Island, N.Y.)
- 10^{-6} M hydrocortisone (Sigma, St. Louis, Mo.)
- 5 uq/ml Vitamin D_3 (U.S. Biochemical Corp., Cleveland, Ohio)
- 20% fetal calf serum, heat-inactivated (Hyclone Laboratories, Logan, Utah)
- 2 gm/l $NaHCO_3$ (Fisher Scientific Co., Fair Lawn, N.J.)
- 100 U/ml Penicillin
- 100 ug/ml Streptomycin
- 0.25 ug/ml Fungizone

Various conditions and factors can be tested for any effect increasing the self-renewal ability of stem cells in vitro. These include but are not limited to oxygen tension (see Smith and Broxmeyer, 1986, Br. J. Hematol. 63:29–34, incorporated by reference herein), superoxide dismutase (Sigma Chemical Co., St. Louis, Mo.), glucose oxidase (Sigma Chemical Co.), and combinations of various colony stimulating factors, namely interleukin-3 (IL-3), granulocyte-macrophage (GM)-colony stimulating factor (CSF), granulocyte (G)-CSF, macrophage (M)-CSF (CSF-1), erythropoietin, IL-1, and IL-4 (B cell growth factor).

6.10. Mouse Dissection Protocols

Mouse bone marrow and spleen are valuable sources of murine hematopoietic stem and progenitor cells for model studies testing new and/or improved protocols for use with the human neonatal stem and progenitor cells of the present invention. Procedures for dissection of mouse bone marrow and spleen are described in Sections 6.10.1, and 6.10.2, respectively.

6.10.1. Bone Marrow Dissection

The following procedure can be used to obtain a murine bone marrow cell suspension:

1. Sacrifice mouse as prescribed by cervical-thoracic dislocation.
2. Inside a laboratory hood, soak the mouse with 70% ethanol (to avoid microbial contamination), completely wetting the fur.
3. Snip through the skin, and peel the skin down to the hip by holding the foot with either forceps that have been soaked in 70% ethanol, or with fingers, and pulling the skin with forceps.
4. With sterile (alcohol-treated) forceps and scissors, cut away as much muscle tissue as possible to expose the femur.
5. Remove the tibia from the femur by cutting through the knee cartilage/joint. Discard the tibia.
6. Remove the femur from the body by placing the sharp edge of a scissors on the anterior side of the hip joint, and pulling the femur in the opposite direction against the scissors, so that the scissors fits in the fold. Snip through the joint.
7. Remove the knee end of the femur first, by snipping just the end with a scissors. Remove the hip end from the femur by the same method.
8. With a 10 cc syringe containing 5 ml media (McCoys 5A 1×) and a 27 gauge needle, place the needle in the bone cavity via the hip end of the bone.
9. Flush the marrow from the bone by forcing media into the cavity with the syringe, while holding the bone and syringe over a 17×100 mm tube.
10. After both femurs have been evacuated, break up clumps with a 10 cc syringe and a 23 gauge needle.
11. Pellet the cells by centrifugation at 400×g (1500 rpm in a Beckman TJ-6R rotor) for 10 minutes at 4° C.
12. Aspirate the supernatant and discard it.
13. Resuspend the cells with 10 ml McCoys 5A media and a pipette, and repeat steps 11 and 12.
14. Resuspend the cells with 10 ml McCoy's 5A media with a pipette, and count the cells (with a hemocytometer).

6.10.2. Spleen Dissection

The following procedure ban be used to obtain a murine spleen cell suspension:

1. sacrifice mouse as prescribed by cervical-thoracic dislocation.
2. Inside a laboratory hood, soak the mouse with 70% ethanol (to avoid microbial contamination), completely wetting the fur.
3. Place the mouse on its abdomen and snip through its left side skin with a sterile scissors and forceps.

4. Lift the peritoneum with the forceps, and snip through to the abdominal cavity.
5. With the spleen in view, remove it and place it in a 60×100 mm dish containing 5–7 ml media.
6. Place the spleen in a sterile homogenizing screen, in the dish, and snip it into small pieces.
7. With the plunger of a 10 cc syringe, gently work the tissue through the screen into a dish containing media.
8. Transfer the cell suspension from the dish to a tube. Rinse the plate with 3 ml media and pool.
9. Resuspend small pieces by transferring the cell suspension from the tube to a 10 cc syringe, and passing it through a 23 gauge needle twice.
10. Pellet the cells by centrifugation at 400×g (1500 rpm) for 10 minutes at 4° C.
11. Aspirate the supernatant and discard it.
12. Resuspend the cells with 10 ml McCoy's 5A media and a pipette, and repeat steps 10 and 11.
13. Resuspend the cells with 10 ml McCoy's 5A media, and count the cells (with a hemocytometer).

6.11. Hematopoietic Reconstitution of Adult Mice with Syngeneic Fetal or Neonatal Stem Cells

The experiments described in the examples sections infra demonstrate the hematopoietic reconstitution of adult mice with syngeneic or Tla-congenic stem cells of fetal or neonatal blood.

A key reference and source of citations for use in animal model studies, which describes standards for experimental irradiation, of mice and other mammals, at the level causing 100% mortality from hematopoietic failure, and prevention of such mortality by hematopoietic reconstitution (with bone marrow cells), is: Balner, H. Bone Marrow Transplantation and Other Treatment after Radiation Injury, Martinus Nijhoff Medical Division, The Hague, 1977, which is incorporated by reference herein.

6.11.1. Hematopoietic Reconstitution of Lethally-irradiated Mice with Stem Cells in Blood of the Near-term Fetus

The examples herein described demonstrate that stem cells in blood of the near-term fetus are able to reconstitute the hematopoietic system of lethally-irradiated mice.

The irradiated mice were ten (B6×A-Tla^b)F_1 hybrid males, aged seven weeks. The mice were exposed to 862.8 rads at a radiation dose of 107.85 rad/min for 8 minutes with a ^{137}Cs source. This dose constitutes the LD100/30 days, i.e., the minimum or near-minimal Lethal Dosage causing 100% mortality within a 30-day post-irradiation period. Use of the 30-day survival endpoint is standard because hematopoietic reconstitution is deemed sufficient by that time, and any later mortality is therefore attributable to causes other than hematopoietic failure.

Blood was collected from five near-term (B6-Tla^a×A)F_1 hybrid fetuses, delivered by Caesarian section from one mother. In this experiment, near-term fetuses were used instead of neonates in order to ensure microbial sterility. The genetics of donor and recipient mice provides complete histocompatibility except for a segment of chromosome 17 bearing the Tla marker gene. All mice were maintained previously and throughout on acidified drinking water to eradicate pseudomonas and similar infective organisms.

As a restorative treatment, three mice each received 0.17 ml heparinized whole fetal blood (made up to a total volume of approximately 0.2 ml by adding M199 medium with penicillin and streptomycin added) by intravascular injection into a peri-orbital vein of the eye, within two hours of irradiation. The results (Table VI) demonstrated the resultant survival of mice reconstituted with fetal blood stem cells, in contrast to the observed death of mice which had undergone no restorative treatment.

TABLE VI

HEMATOPOIETIC RECONSTITUTION OF LETHALLY-IRRADIATED ADULT MICE WITH STEM CELLS IN BLOOD OF THE NEAR-TERM FETUS

Group	Day of Death	30-day Survival Rate*
(1) Treated	14	2/3**
(2) Controls: no restorative treatment but conditions otherwise identical	11, 12, 12, 13, 13, 15, 15	0/7

*All 30-day survivors were normally healthy over prolonged periods of observation, displaying the typical post-irradiation graying of the coat, and would doubtless have experienced an approximately normal life-span, as is typical of reconstitution with syngeneic or near-syngeneic cell donors.
**Later typing for the Tla marker by cytotoxicity assay of thymocytes (Schlesinger, M., et al., 1965, Nature 206:1119–1121; Boyse, E. A., et al., 1964, Methods in Medical Research 10:39) established repopulation by donor cells of the injected blood.

6.11.2. Hematopoietic Reconstitution of Mice with a Lesser Volume of Near-term Fetal Blood but not with Adult Blood

In the example herein, we describe a procedure which was carried out to effect the hematopoietic reconstitution of a patent by allogeneic peripheral blood stem cell infusion, for treatment of the genetic anemia Fanconi's syndrome.

The examples herein described demonstrate that a defined volume of near-term fetal blood contains adequate hematopoietic stem cells to effectively reconstitute the hematopoietic system of lethally-irradiated mice, while the same volume of adult blood will not effect successful reconstitution.

The irradiated mice were 20 (B6×A-Tla^b)F_1 hybrid males aged 7 weeks, and 10 (B6×A-Tla^b)F_1 females aged 7 weeks. The mice were exposed to 862.8 rads at a radiation dose of 107.85 rad/min for 8 minutes with a ^{137}Cs source (LD100/30 days).

Blood was collected from eight near-term (B6-Tla^b×A)F_1 hybrid fetuses, delivered by Caesarian section from one mother. In this experiment, near-term fetuses were used instead of neonates in order to ensure microbial sterility. The genetics of donor and recipient mice provides complete histocompatibility except for a segment of chromosome 17 bearing the Tla marker gene. All mice were maintained previously and throughout on acidified drinking water to eradicate pseudomonas and similar infective organisms.

As a restorative treatment, 10 mice received 0.02 ml heparinized whole fetal blood per mouse (made up to a total volume of 0.22 ml by adding M199 medium with penicillin and streptomycin added), and 10 mice each received 0.02 ml adult whole blood identically treated, by intravascular injection into a peri-orbital vein of the eye, within 2 hours of irradiation. Control mice received no restorative treatment. The results (Table VII) demonstrated that stem cells in a defined volume of fetal blood can successfully reconstitute the hematopoietic system, while cells in an equal volume of adult blood cannot.

TABLE VII

SUCCESSFUL HEMATOPOIETIC RECONSTITUTION WITH A DEFINED VOLUME OF NEAR-TERM FETAL BLOOD BUT NOT WITH ADULT BLOOD

Group	Day of Death	30-day Survival Rate*
(1) Treated with fetal blood	10, 12, 12, 14, 14	5/10**
(2) Treated with adult blood	11, 11, 12, 12, 12, 13, 14, 14, 15, 15	0/10
(2) Controls: no restorative treatment but conditions otherwise identical	9, 10, 10, 11, 11, 12, 12, 12, 15, 23	0/10

*All 30-day survivors were normally healthy over prolonged periods of observation, displaying the typical post-irradiation graying of the coat, and would doubtless have experienced an approximately normal life-span, as is typical of reconstitution with syngeneic or near-syngeneic cell donors.
**Later typing for the Tla marker by cytotoxicity assay of thymocytes (Schlesinger, M., et al., 1965, Nature 206:1119–1121; Boyse, E. A., et al., 1964, Methods in Medical Research 10:39) established repopulation by donor cells of the injected blood.

6.11.3. Hematopoietic Reconstitution with Blood of Newborn Mice in Volumes as Low as Ten Microliters

The examples herein described demonstrate that the stem cells in a volume of neonatal blood as low as 10 microliters can reconstitute the hematopoietic system of lethally-irradiated mice.

The irradiated mice were 20 (B6×A-Tla^b)F$_1$ hybrid males aged 8–12 weeks. The mice were exposed to 862.8 rads at a radiation dose of 107.85 rad/min for 8 minutes with a ^{137}Cs source (LD100/30 days).

Blood was collected by cervical section from eighteen (B6-Tla^a×A)F$_1$ hybrid neonates, less than 24 hours old. As a restorative treatment, 5 mice received 0.04 ml heparinized whole neonatal blood per mouse (made up to a total volume of approximately 0.2 ml by adding M199 medium with penicillin and streptomycin added), (Group 1); 5 mice each received 0.02 ml (Group 2); 5 mice each received 0.01 ml (Group 3); and 5 mice received no further treatment (Group 4, radiation control). Treatment was by intravascular injection into a peri-orbital vein of the eye.

The genetics of donor and recipient mice provides complete histocompatibility except for a segment of chromosome 17 bearing the Tla marker gene. All mice were maintained previously and throughout on acidified drinking water to eradicate pseudomonas and similar infective organisms.

The results in Table VIII show that stem cells in extremely small neonatal blood volumes (down to 10 ul) were able to reconstitute the hematopoietic system.

TABLE VIII

SUCCESSFUL HEMATOPOIETIC RECONSTITUTION WITH NEONATAL BLOOD VOLUMES AS LOW AS TEN MICROLITERS

Group	Day of Death	30-day Survival Rate*
(1) Treated with 0.04 ml neonatal blood	12	4/5**
(2) Treated with 0.02 ml neonatal blood	14, 18	3/5
(3) Treated with 0.01 ml neonatal blood	12, 12, 14, 14	1/5

TABLE VIII-continued

SUCCESSFUL HEMATOPOIETIC RECONSTITUTION WITH NEONATAL BLOOD VOLUMES AS LOW AS TEN MICROLITERS

Group	Day of Death	30-day Survival Rate*
(4) Controls: no restorative treatment but conditions otherwise identical	5, 6, 9, 10, 11	0/5

*All 30-day survivors were normally healthy over prolonged periods of observation, displaying the typical post-irradiation graying of the coat, and would doubtless have experienced an approximately normal life-span, as is typical of reconstitution with syngeneic or near-syngeneic cell donors.
**Later typing for the Tla marker by cytotoxicity assay of thymocytes (Schlesinger, M., et al., 1965, Nature 206:1119–1121; Boyse, E. A., et al., 1964, Methods in Medical Research 10:39) established repopulation by donor cells of the injected blood.

6.11.4. Hematopoietic Reconstitution with Blood of Newborn Mice in Volumes of 10 or 15 Microliters

The examples herein described demonstrate that the stem cells in a volume of neonatal blood as low as 10 or 15 microliters can reconstitute the hematopoietic system of lethally-irradiated mice.

The irradiated mice were 15 male and 5 female (B6×A-Tla^b)F$_1$ hybrids aged 10–12 weeks. The mice were exposed to 862.8 rads at a radiation dose of 107.85 rad/min for 8 minutes with a ^{137}Cs source (LD100/30 days).

Blood was collected by cervical section from fourteen (B6×A-Tla^b)F$_1$ hybrid neonates, less than 24 hours old. As a restorative treatment, 10 mice received 0.015 ml heparinized whole neonatal blood per mouse (made up to a total volume of approximately 0.2 ml by adding M199 medium with penicillin and streptomycin added), (Group 1); 5 mice each received 0.01 ml (Group 2); and the 5 female mice received no further treatment (Group 3, radiation control). Treatment was by intravascular injection into a peri-orbital vein of the eye. The donor and recipient mice were genetically identical, and thus completely histocompatible. All mice were maintained previously and throughout on acidified drinking water to eradicate pseudomonas and similar infective organisms.

The results shown in Table IX reveal that stem and progenitor cells in neonatal blood volumes of 10 or 15 microliters were able to reconstitute the hematopoietic system.

TABLE IX

SUCCESSFUL HEMATOPOIETIC RECONSTITUTION WITH NEONATAL BLOOD VOLUMES OF 10 OR 15 MICROLITERS

Group	Day of Death	30-day Survival Rate*
(1) Treated with 0.015 ml neonatal blood	12, 12, 12, 13, 13, 13	4/10
(2) Treated with 0.01 ml neonatal blood	12, 16	3/5
(4) Controls: no restorative treatment but conditions otherwise identical	12, 13, 14, 17, 22	0/5

*All 30-day survivors were normally healthy over prolonged periods of observation, displaying the typical post-irradiation graying of the coat, and would doubtless have experienced an approximately normal life-span, as is typical of reconstitution with syngeneic or near-syngeneic cell donors.

6.12. Successful Hematopoietic Engraftment and Repopulation by Cryopreserved HLA-identical Umbilical Cord Blood from a Sibling in a Case of Fanconi Anemia

In the example herein, we describe a procedure which was carried out to effect the hematopoietic reconstitution of a patient by allogeneic peripheral blood stem cell infusion, for treatment of the genetic Fanconi anemia syndrome.

6.12.1. Fanconi Anemia

Fanconi anemia (FA) is an autosomal recessive disorder that entails progressive pancytopenia and predisposition to malignancy together with non-hematopoietic developmental anomalies (Fanconi, G., 1967, Semin. Hematol. 233–240; Schroeder, T. M., 1976, Hum. Genet. 32:257–288; Swift, M., 1971, Nature 230:270–373). The FA phenotype is variable so that diagnosis by clinical manifestations alone is difficult, (Glanz, A. et al., 1982, J. Med. Genet. 19:412–416; Auerbach, A. D., 1989, Blood 73:391–396) but hypersensitivity to the clastogenic effect of DNA crosslinking agents such as diepoxybutane (DEB) or nitrogen mustard provides a diagnostic indicator of the FA genotype both prenatally and postnatally (Auerbach, A. D., 1989, Blood 73:391–396; Auerbach, A. D., 1976, Nature 261:494–496; Berger, R. et al., 1980, Cancer Genet. Cytogenet. 2:269–274; Auerbach, A. D. et al., 1981, Pediatrics 67:128–135; Auerbach, A. D. et al., 1985, Pediatrics 76:794–800; Auerbach, A. D. et al., 1986, Hum. Genet. 73:86–88).

Patients with FA often die young from complications of bone marrow aplasia such as hemorrhage or infection, or from leukemia. The present ability to perform HLA typing on fetal cells obtained during the first or second trimester of preganancy, (Calloway, et al., 1986, Human Immunol. 16:200–204) makes it possible to ascertain whether a fetus is HLA-identical to a sibling affected with FA (Auerbach, A. D., 1988, Blood 62(Suppl 1):53a).

The example herein describes the successful hematopoietic reconstitution of a boy with severe Fanconi anemia by means of cryopreserved umbilical cord blood of a sister shown by prenatal testing to be unaffected with Fanconi anemia, karyotypically normal, and HLA identical to the patient. The availability of cord blood in this case obviated the need for bone marrow aspiration from the infant sibling.

6.12.2. Patient Characteristics

The patient, a five year-old male, was first noted to be pancytopenic at 24 months of age. Evaluation at Duke University Medical Center showed the following initial blood counts: hemoglobin 9.5 g per 100 ml, leucocytes 4.3×10^9 per liter with 22% granulocytes, 62% lymphocytes, 3% monocytes, 1% eosinophils, 8% basophils, platelets 62×10^9 per liter and 2.3% reticulocytes. The bone marrow was hypocellular. The patient had classical malformations of Fanconi anemia, with retarded growth of 5th to 10th percentile, a left extra rudimentary thumb, an absent left kidney and hypospadias. Testing of patient's cells with diepoxybutane (DEB) (DEB induced chromosomal breakage assay; Auerbach et al., 1979, Am. J. Hum. Genet. 31(1):77–81) performed at 24 months at the Rockefeller University, confirmed the diagnosis of Fanconi anemia. The parents were healthy and there was no consanguinity or past history of blood disorders in the family. The patient had been treated with Danazol 300 mg/day for 6 months with a progressive fall of blood counts. Before admission, he had received 3 units of filtered irradiated packed red blood cells.

The mother became pregnant. Cytogenetic analysis of cultured amniotic fluid cells at the Rockefeller University showed that baseline and DEB-induced breakage were within the normal range (Auerbach, A. D., et al., 1985, Pediatrics 76:794–800). Chromosome breakage studies on cord blood indicated that the newborn was karyotypically normal and not affected with Fanconi anemia (Table X). HLA typing of amniotic fluid cells, by Dr. M. S. Pollack, the Methodist Hospital and Baylor Medical Center, Houston, TX, showed that the fetus was HLA-identical to the patient: first haplotype HLA-A1, -B8, -DR3; second haplotype HLA-A29, -B44, -DR3. Mixed lymphocyte cultures of patient cells with cord blood and donor peripheral blood lymphocytes were negative. The ABO blood group of the donor was O Rh+and that of the recipient B Rh+.

At the time of admission in September 1988, the patient's blood showed hemoglobin 6.8 g per 100 ml, leucocytes 3.1×10^9 per liter, granulocytes 7%, lymphocytes 86%, monocytes 5% and platelets 18×10^9 per liter. The marrow was hypocellular with 10% of normal cellularity, 24% myeloid cells, 2% erythroblast cells, 67% lymphocytes and no megakaryocytes. There were no hemorrhaggic or infectious complications. Liver and kidney functions were normal, CMV serology was positive by ELISA. Serology for HIV, hepatitis B and toxoplasmosis was negative.

The patient was isolated in a landmark flow room and given oral broad spectrum non-absorbable antibiotics (Vancomycine, Tobramycine, and Colimycine) starting on day 8. Fluconazole (50 mg/day) was administered for prevention of fungal infection and oral acyclovir (100 mg/day) for prevention of herpes simplex virus infection. A pretransplant conditioning procedure developed specifically for Fanconi anemia was used (Gluckman, E. et al., 1989, in Fanconi Anemia, Clinical Cytogenetic and Experimental Aspects, Auerbach and Obe, eds., Springer Verlag, Berlin, pp. 60–68) that accommodates the hypersensitivity of Fanconi cells to alkylating agents which crosslink DNA (Gluckman, E. et al., 1980, Brit. J. Haematol. 45:557–564; Berger, R. et al., 1980, Brit. J. Haematol. 45:564–568) and to irradiation (Gluckman, E., et al., 1983, Brit. J. Haematol. 54:431–440). The patient was conditioned with cyclophosphamide 5 mg/kg intravenous (i.v.) for four consecutive days from day −6 to day −3 (total dose received was 380 mg) with hyperhydration. On day −1, he received 5 Gy thoracoabdominal irradiation delivered by a linear accelerator, at a mean dose rate of 10.87 cGY/min. Total duration of irradiation was 46 minutes. The dose delivered to the abdomen was 500 cGy. The lungs and the right liver lobe were shielded and received 67 cGy.

On day 0, cryopreserved cord blood was thawed and infused without further processing according to predetermined optimal conditions (Broxmeyer, HE et al., 1989, Proc. Natl. Acad. Sci. USA 86:3828–3821) (surviving whole red cells not already hemolyzed being group 0). The patient received 0.4×10^8 nucleated cells/kg, of which a total of 4.37×10^5 were CFU-GM. Two hours after the infusion, the patient had chills, fever, and hypotension. These symptoms soon resolved, the patient receiving broad spectrum antibiotics Vancomycine and Ceftazidine i.v. For graft-versus-host disease prophylaxis, Cyclosporine A was administered i.v. from day −1 at a dose of 4.5 mg/kg/day according to a preliminary pharmacokinetic study. All blood products were irradiated at 25 Gy.

TABLE X

CYTOGENETIC ANALYSIS

Source of Cells	Days Post Transplant	% Female Cells[a] Baseline	% Female Cells[a] DEB-Treated[c]	Mean Chromosome Breaks/Cell[b] Baseline	Mean Chromosome Breaks/Cell[b] DEB-Treated[c]
Pre-Transplant Peripherial Blood	0	0	0	0.18	10.6
Donor Cord blood	0	100	100	0.00	0.02
Bone marrow	120	100	100	0.04	0.30
Peripheral blood	50	30	52	0.7	4.0
Peripheral blood	64	8	32	1.3	5.0
Peripheral blood	120	12	32	1.9	6.4
Peripheral blood	204	64	86	0.30	0.49

[a]Analysis performed on quinicrine-stained metaphase preparations.
[b]Analysis performed on Giemsa-stained metaphase preparations.
[c]Final concentration of DEB in the medium was 0.1 mg/ml.

6.12.3. Ethical and Regulatory Considerations

Written informed consent was obtained from the patient's family for the collection of cord blood and for the transplantation procedure. The treatment plan was reviewed and approved by the Institutional Review Board for Clinical Investigation of the Duke University Medical Center and by the Ethical Committee of the Hopital Saint Louis. Approval for receipt, cryopreservation, storage and release of cord blood was received from the Institutional Review Board or the Indiana University School of Medicine. The U.S. Food and Drug Administration considered the procedure equivalent to bone marrow storage and transplantation, which are currently not subject to FDA regulation.

6.12.4. Methods

6.12.4.1. Cytogenetic Studies

Chromosome breakage studies were performed as described (Auerbach, A. D. and Wolman, S. R., 1976, Nature 261:494–496). DEB was added to bone marrow cultures at the time of initiation and cells harvested after 24 hours. Peripheral blood was cultured in the presence of phytohemagglutinin (PHA) for 72 hours, with DEB present in the medium for the last 48 hours of culture. The frequency of baseline and DEB-induced chromosomal breakage was analyzed using Giemsa-stained metaphase preparations; the ratio of male to female cells was also determined on quinicrine-stained slides to facilitate the identification of the Y chromosome.

6.12.4.2. DNA Studies:Restriction Fragment Length Polymorphism

DNA samples for Southern blotting were digested with TaqI (New England Biolabs), separated by gel electrophoresis, transferred to an Immobilon-N™ filter (Millipore Corporation, Bedord, Mass.), hybridized, and washed as described (Mann, W., et al., 1989, Nucleic Acids Res. 17:5410). The probe used was CRI-pS232 (DXS278), (Collaborative Research, Boston, Mass.), which hybridizes with sequences from the X and Y chromosomes.

6.12.4.3. Collection, Storage and Shipment of neonatal Blood

Immediately upon uncomplicated vaginal delivery of the sibling infant, blood was collected from the umbilical cord and from the placenta as described (Broxmeyer, H. E., et al., 1989, Proc. Natl. Acad. Sci. USA 86:3828–3832; see supra) and sent at ambient temperature by overnight express service to the Indiana University School of Medicine for cellular analysis, cryopreservation and storage. A sample of cord blood was also sent to the Rockefeller University for cytogenetic analysis. After removing a small sample (<2 ml) for laboratory tests, including determination of nucleated cellularity and enumeration of progenitor cells, the blood was frozen without further manipulation in a final concentration of 10% v/v dimethyl sulphoxide (DMSO) as described (Broxmeyer, H. E. et al., 1989, Proc. Natl. Acad. Sci. USA 86:3828–3832; see supra). Two bags of cord blood and 1 bag of placental blood were frozen. Sample volumes (1 ml each in Nunc tubes) were similarly frozen for tests of recovery after thawing and for confirmation of HLA type. The volume of blood and numbers of nucleated and progenitor cells collected are given in Table XI. These numbers are within the range associated with successful HLA-matched allogeneic bone marrow transplantation (Broxmeyer, H. E., et al., 1989, Proc. Natl. Acad. Sci. USA 86:3828–3832). Test thaws, including one tube sent from Indiana University to Duke University Medical Center (where HLA type was confirmed), yielded 79% to 90% recovery of nucleated cells with recoveries of 100% of day 14 CFU-GM, 63±18% of BFU-E and 79±15% of CFU-GEMM.

With airline approval, the 2 bags of cord blood and the 1 bag of placental blood were escorted by air to the Hopital Saint-Louis from Indiana University 2 weeks before the transplant. The frozen bags were sent in a Dry Shipper (CMC-3200 wide mouth with platform, Cryomed, New Baltimore, Mich.) that maintains temperature at −175° C., which is optimal for cryoprotection.

82% of nucleated cells of the thawed blood transfused into the patient were viable. Progenitor cell assays in Paris (differing slightly in technique from assays in Indiana) indicated recovery equal to or greater than counts of progenitors before freezing.

TABLE XI

Numbers of Hematopoietic Progenitor Cells in Blood from Umbilical Cord and Placenta and In Recipient Bone Marrow Immediately Prior to Conditioning and 30 and 120 Days Post-Transplant[a]

	Unseparated	Low Density (<1.077 gm/ml)
Total Hematopoietic Progenitor Cells in Cord Blood × 10^{-3}[b]		
a) Agar Culture		
Day 14 CFU-GM (colonies)	1.52	ND
Day 14 CFU-GM (colonies & clusters)	2.46	ND

TABLE XI-continued

Numbers of Hematopoietic Progenitor Cells in Blood from Umbilical Cord and Placenta and In Recipient Bone Marrow Immediately Prior to Conditioning and 30 and 120 Days Post-Transplant[a]

	Unseparated	Low Density (<1.077 gm/ml)
b) Methyl Cellulose Culture (colonies)		
Day 14 CFU-GM	1.56	ND
BFU-E-2	3.95	ND
BFU-E-1	3.60	ND
CFU-GEMM	0.39	ND
Progenitors in Recipient Bone Marrow per 10^5 Cells Plated		
1) Pre-Transplant Methylcellulose Culture		
Day 14 CFU-GM (colonies)	ND	1
Day 14 CFU-GM (colonies & clusters)	ND	8[c]
BFU-E-1	ND	0
CFU-GEMM	ND	0
2) Day 30 Post-Transplant Methylcellulose culture		
Day 14 CFU-GM (colonies)	ND	9
BFU-E-1	ND	0
CFU-GEMM	ND	0
3) Day 120 Post-Transplant		
a) Agar Culture		
Day 7 CFU-GM (colonies)	22 ± 2	140 ± 12
Day 7 CFU-GM (colonies & clusters)	118 ± 16	416 ± 20
Day 14 CFU-GM (colonies)	36 ± 4	156 ± 8
Day 14 CFU-GM (colonies & clusters)	152 ± 4	228 ± 12
b) Methyl Cellulose Culture (colonies)		
Day 14 CFU-GM	324 ± 16	416 ± 25
BFU-E-2	77 ± 2	134 ± 6
BFU-E-1	96 ± 16	132 ± 10
CFU-GEMM	4 ± 1	9 ± 1

a. Results are expressed as mean±1 S.E.M. Progenitor cell assays were set up as described in elsewhere (Broxmeyer, H. E. et al., 1989, Proc. Natl. Acad. Sci. USA 86:3828–3832). The cord blood and 120 post-transplant cultures were done in Indiana, while the pre-transplant and day 30 post-transplant studies were done in Paris. The frequency of hematopoietic progenitors per 10^3 low density bone marrow cells plated from healthy normal donors ranges from 10 to 70 for CFU-GM and from 15 to 80 for BFU-E-2 and BFU-E-1 at the Lab in Indiana.

b. The total volume of cord and placental blood collected was 160 ml and contained 1.19×10^9 nucleated cells.

c. These were mainly microclusters, <20 cells/cluster.

ND, not done.

6.12.4.4. Hematopoietic Progenitor Cells in Vivo

Assays were set up as described (Broxmeyer, H. E., et al., 1989, Proc. Natl. Acad. Sci. USA 86:3828–3832). For the CFU-GM assay, colonies (>40 cells/aggregate) and clusters (3–40 cells/aggregate) were scored after 7 days and 14 days of incubation in agar culture medium. Large-sized colonies formed (>1000 cells). Results are expressed also as colonies plus clusters, which more accurately reflects the total CFU-GM compartment. For the BFU-E, CFU-GEMM, and CFU-GM assays, colonies were scored after 14 days of incubation in methylcellulose culture medium. BFU-E-1, CFU-GEMM, and CFU-GM assays were scored from the same plates, which included erythropoietin (1 unit/ml), hemin (0.1 mM), and 5637 conditioned medium (DM) (10% v/v). BFU-E-2 were cultured as BFU-E-1 but without 5637CM. BFU-E-2 colonies contained at least 50 cells or comprised at least three subcolonies which contained at least 10 cells, but were usually much larger. Colonies derived from BFU-E-1 were much larger than those derived from BFU-E-2.

6.12.5. Results

6.12.5.1. Clinical

The clinical course was uneventful without complications. Conditioning was tolerated without evident toxicity. On day 15, a transient skin rash and fever resolved with 2 mg/kg methylprednisolone. Skin biopsy showed few vacuolar basal epidermal cells with mild lymphoid infiltrate defined as a grade I GVH according to Seattle classification (Sale, G. E., et al., 1977, Am. J. Pathol. 89:621–633). Liver function tests showed a rise of SGOT and SGPT (×2N) probably related to GVH, returning to normal on day 47. CMV was repeatedly isolated from the urine but the patient never had signs of active infection and all tests for viremias were negative. Five months after transplant the patient was discharged with normal clinical and biological findings. Cyclosporine A and corticosteroids were progressively reduced and discontinued at 6 months. At 9 months after transplantation, the patient has no chronic graft versus host disease and leads a normal life.

6.12.5.2. Hematological

Recovery of blood counts is shown in Table XII. Reticulocytes and granulocytes began to rise by day 22 after cord blood transplantation. The patient received 8 transfusions of packed RBC (O Rh+) and 48 units of random platelets. RBC were last transfused on day 54 and platelets on day 43. Bone marrow aspirated on day 17 was aplastic and on day 28 showed 20% cellularity with 19% myeloid cells, 73% erythroid cells and few megakaryocytes. On day 120, the marrow showed normal cellularity with 40% myeloid cells, 44% erythroid cells and normal megakaryocytes.

TABLE XII

Blood Counts After Cord Blood Transplantation

Days	Hemoglobin	Leucocytes	Granulocytes	Lymphocytes	Platelets	Reticulocyte
-20	6.8	3.1	0.25	2.8	18	10
0	9.7	0.8	0	0.8	120	0
8	10.9	0.4	0	0.4	80	0
15	11.6	0.4	0	0.4	39	0
22	7.8	0.9	0.3	0.6	50	5
29	8.5	1.0	0.3	0.5	105	17
36	9.4	1.7	0.6	0.5	55	36
43	11.3	5.1	2.4	1.9	31	90
50	8.9	3.4	1.5	0.7	62	162
57	8.9	5.6	3.2	1.0	174	63

TABLE XII-continued

Blood Counts After Cord Blood Transplantation

Days	Hemoglobin	Leucocytes	Granulocytes	Lymphocytes	Platelets	Reticulocyte
90	11.3	5.1	4.0	1.1	296	50
120	13	3.9	2.3	1.1	265	40
160	12	3.7	1.4	1.6	293	45
240	12.3	5.2	2.7	1.6	354	50
282	12.2	4.8	2.3	1.2	315	—

Hemoglobin (Hb) is expressed in g per dl, leucocytes, granulocytes, lymphocytes, platelets, and reticulocytes are expressed as numbers in 10^9 per liter.

6.12.5.3. Hematopoietic Progenitor Cells

Prior to the transplant, the marrow showed few or no detectable progenitor cells (Table XI). At 30 days post-transplant, CFU-GM, but not BFU-E or CFU-GEMM were detected; by 120 days post transplant, normal to supranormal frequencies (colonies/clusters per number of cells plated) of progenitors were apparent.

6.12.5.4. Reconstitution by Donor Cells

6.12.5.4.1. Blood Type Studies

The patient's B Rh+ red blood c ell s disappeared progressively and were undetectable on day 90 after transplant; 46 days after the last transfusion 100% of red cells were of donor type (O Rh+) and remain of donor type 240 days after transplant.

6.12.5.4.2. Cytogenetics

Table X shows the results of cytogenetic studies of bone marrow aspirated on day 120 and peripheral blood drawn on days 50, 64, 120, and 204 after transplant. The chromosomal complement of the bone marrow was 46, XX. No male metaphases were seen in 50 quinicrine-stained or 100 Giemsa-stained cells analyzed. Chromosome breakage frequencies were 0.04 and 0.30 breaks per cell in baseline and DEB-treated cultures respectively.

Through day 120 post-transplant, more than 50% of the metaphases seen in cytogenetic preparations from PHA-stimulated peripheral blood cultures were of host origin, as demonstrated by the presence of a Y chromosome. These cells, of lymphoid origin, exhibited highly elevated baseline chromosomal breakage, and severe radiation damage in the form of multiple dicentrics, rings, and chromosomal fragments. These male cells were also hypersensitive to the clastogenic effect of DEB, showing multiple chromatid breaks and exchanges typical of Fanconi anemia. Female cells did not exhibit elevated levels of chromosomal breakage. By day 204 (6 ½ months) post-transplant, the majority of peripheral blood lumphocytes were of donor origin.

These findings signify engraftment of the male recipient with female donor cells, with a minor population of radiation-damaged host cells surviving in the blood. This situation is similar to that reported by others in bone marrow transplantation (Butturini, A., et al., 1986, Blood, 68:954–956).

6.12.5.4.3. DNA Studies

The CRI-pS232 probe recognizes a complex set of fragments at a highly polymorphic locus on the X chromosome as well as a polymorhic locus on the Y chromosome (Knowlton, R. G., et al., 1989, Nucleic Acids Res, 17:423–437). All of the variable bands present in the donor DNA were seen in the DNA extracted from post-transplant peripheral blood samples from the recipient (FIG. 5). In addition, the X and Y alleles of the recipient were seen as faint bands, indicating some chimerism in the peripheral blood with primarily donor cells present.

6.12.6. Discussion

The clinical and biological data presented here signify that surplus cord blood of a single individual is sufficient for hematopoietic reconstitution. Virtually complete occupation of the male patient's myeloid system by female sibling donor cells is indicated by cytogenetics, lack of undue chromosomal fragility, ABO typing and studies of DNA polymorphisms. At 1.5 years post-transplant, greater than 98% of the circulating lymphoid cells are of donor origin.

Subsequent to the experiments presented above, two other HLA-identical sibling cord blood transplants have been successfully carried out for treatment of Fanconi's anemia.

The present success with allogeneic cord blood is particularly significant because animal studies have shown that hematopoietic restoration requires fewer reconstituting cells when these are syngeneic rather than allogeneic (Balner, H., Bone Marrow Transplantation and Other Treatment After Radiation Injury. Martinus Nijhoff Medical Division—the Hague 1977:29–31).

Thus, human cord blood is an efficacious source of sufficient cells for clinical hematopoietic reconstitution.

6.13. Flowchart: Description of a Service

In a particular embodiment of the invention, the isolation and preservation of neonatal hematopoietic stem and progenitor cells is envisioned as a service offered to each prospective cell donor, which can comprise the steps listed below. The description is meant for illustrative purposes only, in no way limiting the scope of the invention.

1. Contact

Initial contact is made between an expectant mother (client) and the obstetrician, who arranges the service.

2. Blood Collection

In the obstetrical ward, after the infant has been delivered and separated from the cord in the usual way, blood is drawn from the cord into a specially designed receptacle, which is sealed and placed in a customized shipping container, together with a data-form, completed by a member of the obstetrical team, giving details of the birth.

3. Transport

Once daily, an overnight freight carrier collects the shipping containers from the obstetrical wards, and transports them to processing headquarters by 10:30 A.M. the following day.

4. Registration

Upon receipt at headquarters, each container is catalogued. The blood enters the laboratory for processing (optional).

5. Blood Processing (optional)

The cells are separated, and the white cells, which include the stem and progenitor cells, are retained for storage.

6. Testing

The separated cells undergo routine testing (see Section 5.1.2, supra). In exceptional cases, special testing may be indicated to determine whether the sample is contaminated, e.g., by maternal blood. Samples may be rejected for reason of contamination or other causes.

7. Packaging and Labeling

Cells from each accepted sample are dispensed into standard freezing vials (cryules) and labeled in conventional and computer-generated characters.

The cells of each individual are allocated to four cryules, two of which are assigned for storage to one freezer and two to another, independently-serviced, freezer. A fifth cryule contains cells set aside for testing of identity, viability, and function, when withdrawal of cells is required for therapy.

Labels are printed by computer, using a special printer, on silk, which withstands immersion in liquid nitrogen. The label data include the registration number, in machine readable and human readable characters, date of freezing, cryule number (1–4, 5) and freezer assignment (A and B).

8. Freezing and Storage

The cryules are subjected to slow freezing, and assigned to two separately maintained liquid nitrogen refrigerators.

9. Permanent Records

The entire preparative history is entered into the permanent records, including location within cryostorage modules. For example, data input for each donor for maintenance in the computer records can comprise:

Registration number
Name
Sex
Date of birth
Place of birth (hospital identification)
Birth certificate number
Name of mother
Date of receipt of cells
Date of freezing
Freezer positions
Obstetrical data
  (a) special circumstances of birth
  (b) if twin, registration number of co-twin
  (c) any health disorder of the mother
Test results
  (a) differential cell counts
  (b) bacterial cultures
  (c) other tests performed

10. Notification to Client

The client is notified of the registration number, for preservation with child's documents, and is asked for information not available at the time of birth (given name, birth number), for inclusion in permanent records.

11. Withdrawal of Cells for Clinical Use Requests for cells for treatment of the donor are made on behalf of the donor by a suitably accredited physician affiliated with an appropriate hospital unit. Cells are withdrawn from the cell bank and matched for identity with the recipient. The cells are also tested for viability and microbial contamination, and quantified in terms of stem cell, progenitor cell, and other categories. Further tests are conducted as required. Cells and an accompanying report are delivered to the medical institution designated by the physician. An appropriate notation is entered in the permanent records.

It is apparent that many modifications and variations of this invention as hereinabove set forth may be made without departing from the spirit and scope thereof. The specific embodiments described are given by way of example only and the invention is limited only by the terms of the appended claims.

Various references are cited herein, the disclosures of which are incorporated by reference in their entireties.

What is claimed is:

1. A cryopreserved pharmaceutical composition comprising:

(a) viable human neonatal or fetal hematopoietic stem cells derived from the umbilical cord blood or placental blood of a single human collected at the birth of said human, in which said cells are present in an amount sufficient to effect hematopoietic reconstitution of a human adult;

(b) an amount of cryopreservative sufficient for cryopreservation of said cells; and

(c) a pharmaceutically acceptable carrier.

2. The composition of claim 1 which further comprises a viable human neonatal or fetal hematopoietic progenitor cell.

3. The composition of claim 1 which comprises whole neonatal or fetal blood.

4. The composition of claim 1 which further comprises an anticoagulent.

5. The composition of claim 1, 2, 3 or 4 in which the cryopreservative comprises dimethyl sulfoxide.

6. A pharmaceutical composition comprising a pharmaceutically acceptable carrier; and human neonatal or fetal hematopoietic stem cells obtained by a method comprising the following steps in the stated order:

(a) isolating human neonatal or fetal blood components containing hematopoietic stem cells derived from the umbilical cord blood or placental blood of a single human at the birth of said human, in which said cells are present in an amount sufficient to effect hematopoietic reconstitution of a human adult;

(b) cryopreserving the blood components in the presence of a cryopreservative with an amount of a cryopreservative sufficient for cryopreservation; and

(c) thawing the blood components, such that the stem cells are viable.

7. The composition of claim 6 which further comprises a human neonatal or fetal hematopoietic progenitor cell.

8. The composition of claim 6 in which the method of obtaining the cells further comprises the step after step (c) of removing a cryopreservative.

9. The composition of claim 6 in which the method of obtaining the cells further comprises the step after step (a) or step (c) of growing the stem cells in vitro.

10. The composition of claim 6 in which the method of obtaining the cells further comprises the step of enriching for stem and progenitor cells by a cell separation procedure.

11. The composition of claim 6 in which the blood components comprise whole neonatal or fetal blood.

12. The composition of claim 6 in which the blood components are isolated by collection from an umbilical cord.

13. The composition of claim 6 in which the blood components are isolated by collection from a placenta.

14. The composition of claim 6 in which the cryopreserving is by use of a cryopreservative.

15. The composition of claim 6 in which the cryopreservative is dimethyl sulfoxide.

16. The composition of claim 6 in which the cryopreserving is by use of liquid nitrogen.

17. A pharmaceutical composition comprising

(a) viable human neonatal or fetal hematopoietic stem cells derived from the umbilical cord blood or placental blood of a single human collected at the birth of said human, in which said cells are present in an amount sufficient to effect hematopoietic reconstitution of a human adult, which cells have been cryopreserved; and

(b) a pharmaceutically acceptable carrier.

18. The composition of claim 17 which further comprises a viable human neonatal or fetal hematopoietic progenitor cell.

19. The composition of claim 17 which comprises whole neonatal or fetal blood.

* * * * *

US006569427B1

(12) **United States Patent**
Boyse et al.

(10) **Patent No.: US 6,569,427 B1**
(45) **Date of Patent: *May 27, 2003**

(54) **ISOLATION AND PRESERVATION OF FETAL AND NEONATAL HEMATOPOIETIC STEM AND PROGENITOR CELLS OF THE BLOOD**

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(58) **Field of Search** 424/529, 530, 424/531, 93.21; 435/240.2

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(57) **ABSTRACT**

The present invention relates to hematopoietic stem and progenitor cells of neonatal or fetal blood that are cryopreserved, and the therapeutic uses of such stem and progenitor cells upon thawing. In particular, the present invention relates to the therapeutic use of fetal or neonatal stem cells for hematopoietic (or immune) reconstitution. Hematopoietic reconstitution with the cells of the invention can be valuable in the treatment or prevention of various diseases and disorders such as anemias, malignancies, autoimmune disorders, and various immune dysfunctions and deficiencies. In another embodiment, fetal or neonatal hematopoietic stem and progenitor cells which contain a heterologous gene sequence can be used for hematopoietic reconstitution in gene therapy. In a preferred embodiment of the invention, neonatal or fetal blood cells that have been cryopreserved and thawed can be used for autologous (self) reconstitution.

56 Claims, 5 Drawing Sheets

Exhibit B

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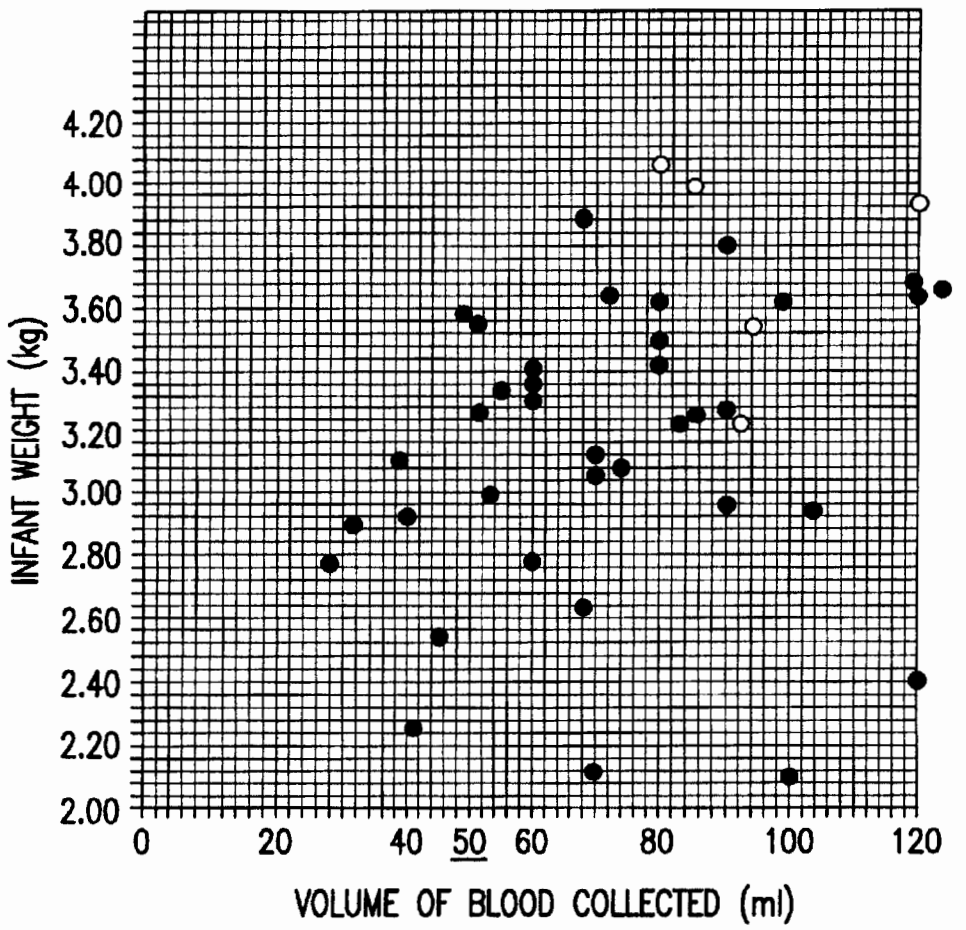
INFANT WEIGHT (kg)
4.20
4.00
3.80
3.60
3.40
3.20
3.00
2.80
2.60
2.40
2.20
2.00
0
20
40
50
60
80
100
120
VOLUME OF BLOOD COLLECTED (ml)

FIG. 1

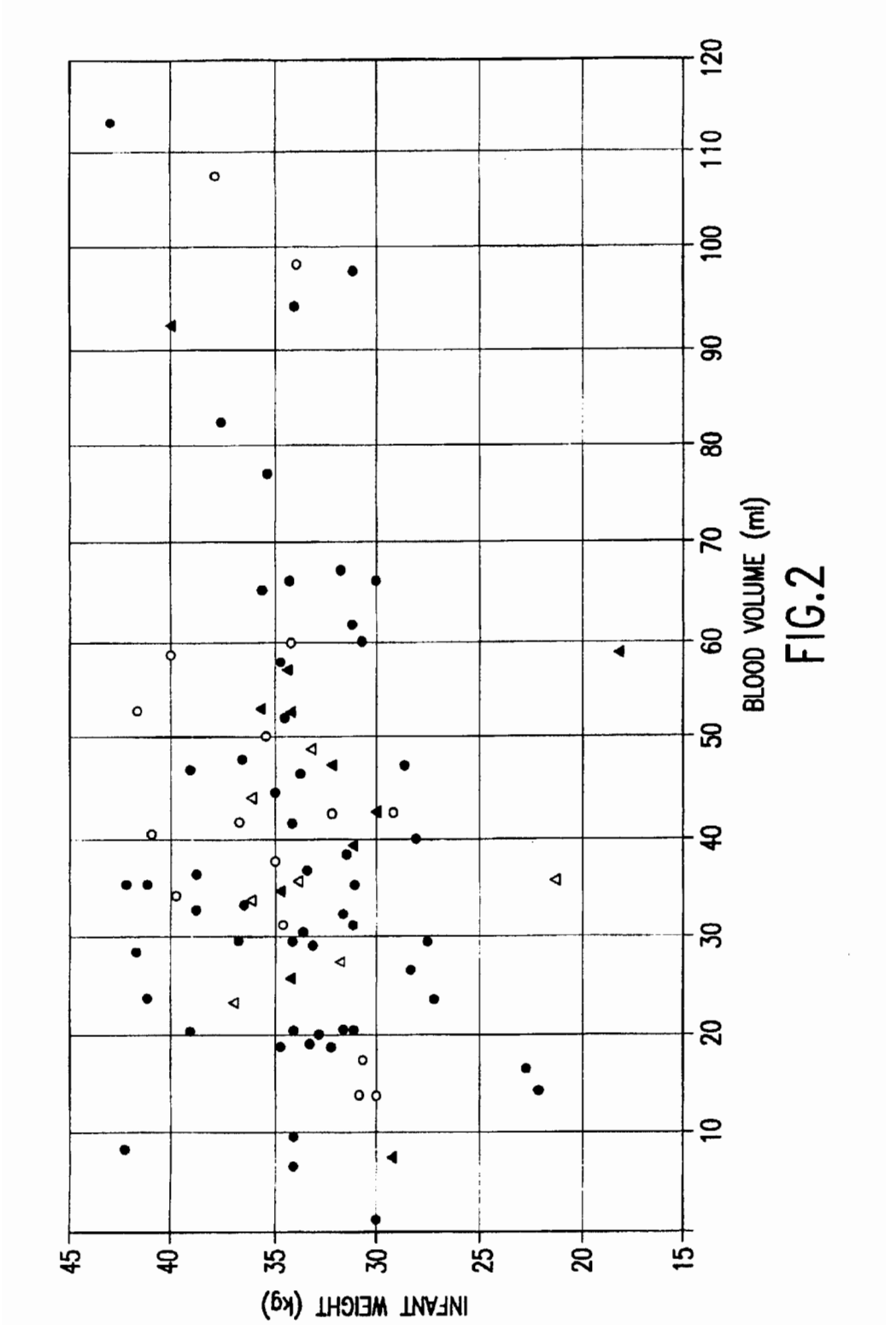
INFANT WEIGHT (kg)
45
40
35
30
25
20
15
BLOOD VOLUME (ml)
10
20
30
40
50
60
70
80
90
100
110
120

FIG.2

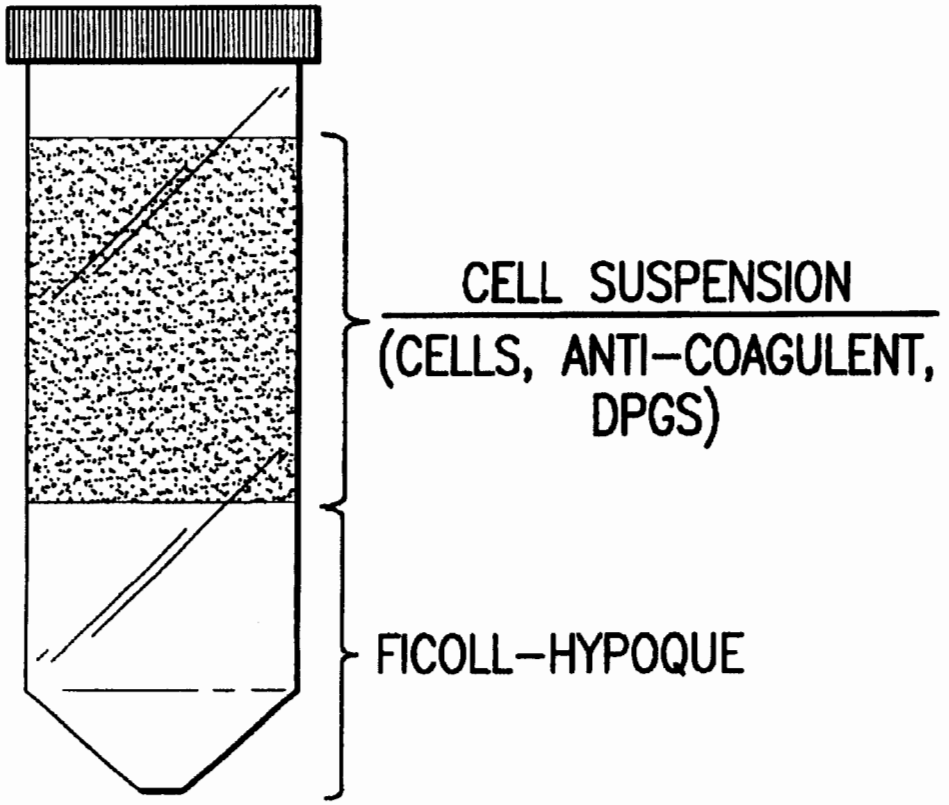

FIG. 3A

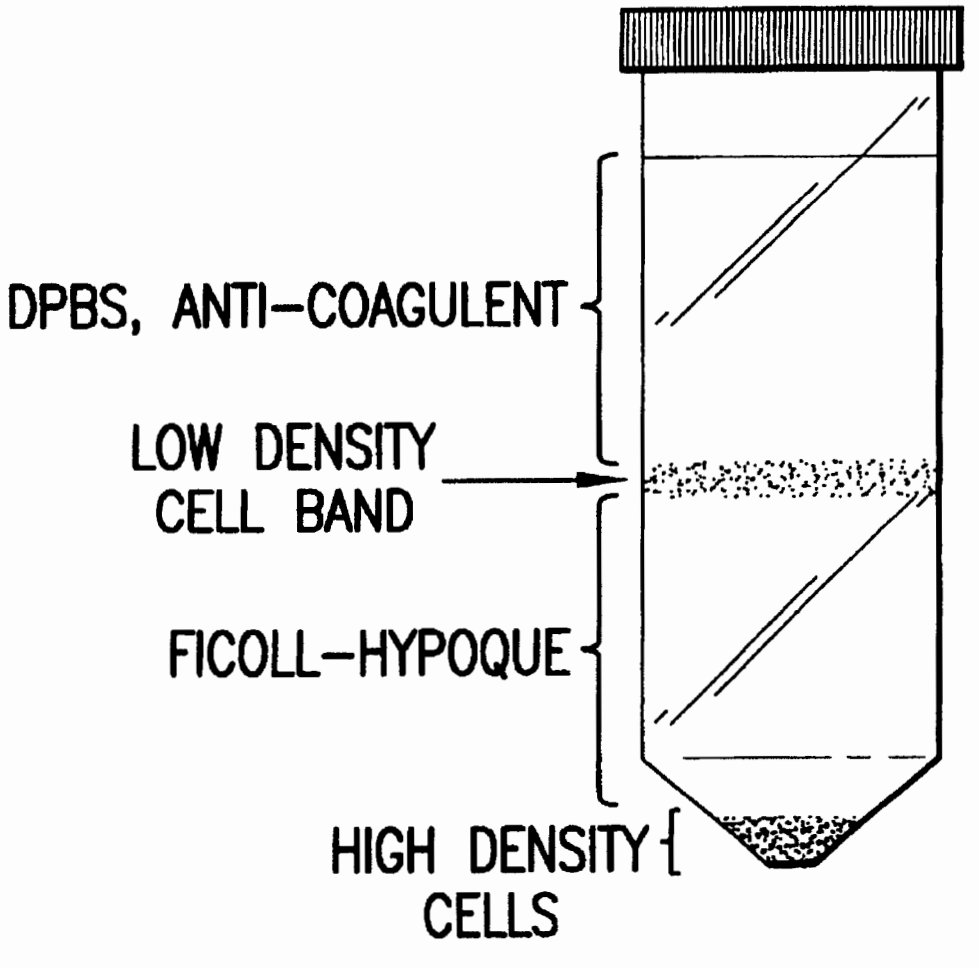
DPBS, ANTI-COAGULENT
LOW DENSITY CELL BAND
FICOLL-HYPOQUE
HIGH DENSITY CELLS

FIG. 3B

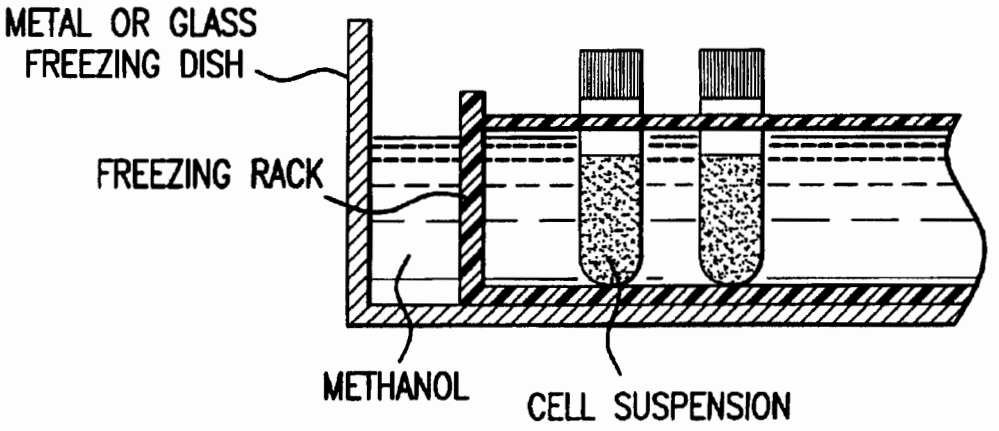
METAL OR GLASS
FREEZING DISH
FREEZING RACK
METHANOL
CELL SUSPENSION

FIG.4

ISOLATION AND PRESERVATION OF FETAL AND NEONATAL HEMATOPOIETIC STEM AND PROGENITOR CELLS OF THE BLOOD

This application is a continuation of application Ser. No. 07/950,356, filed Sep. 24, 1992, now abandoned, which is a continuation of application Ser. No. 07/269,926 filed Nov. 10, 1988 now U.S. Pat. No. 5,192,553, which is a continuation-in-part of application Ser. No. 07/119,746, filed Nov. 12, 1987, now U.S. Pat. No. 5,804,601, each of which is incorporated by reference herein in its entirety.

TABLE OF CONTENTS

1. INTRODUCTION

The present invention is directed to hematopoietic stem and progenitor cells of neonatal or fetal blood, that are

cryopreserved, and the therapeutic uses of such stem and progenitor cells upon thawing. Such cells can be therapeutically valuable for hematopoietic reconstitution in patients with various diseases and disorders. In a preferred embodiment, neonatal cells that have been cryopreserved and thawed, can be used for autologous (self) hematopoietic reconstitution.

The invention also relates to methods for collection and cryopreservation of the neonatal and fetal stem and progenitor cells of the invention.

2. BACKGROUND OF THE INVENTION

2.1. Hematopoietic Stem and Progenitor Cells

The morphologically recognizable and functionally capable cells circulating in blood include erythrocytes, neutrophilic, eosinophilic, and basophilic granulocytes, B-, T-, nonB-, non T-lymphocytes, and platelets. These mature cells derive from and are replaced, on demand, by morphologically recognizable dividing precursor cells for the respective lineages such as erythroblasts for the erythrocyte series, myeloblasts, promyelocytes and myelocytes for the granulocyte series, and megakaryocytes for the platelets. The precursor cells derive from more primitive cells that can simplistically be divided into two major subgroups: stem cells and progenitor cells (for review, see Broxmeyer, H. E., 1983, "colony Assays of Hematopoietic Progenitor Cells and Correlations to Clinical Situations," CRC Critical Reviews in Oncology/Hematology 1(3):227–257). The definitions of stem and progenitor cells are operational and depend on functional, rather than on morphological, criteria. Stem cells have extensive self-renewal or self-maintenance capacity (Lajtha, L. G., 1979, Differentiation 14:23), a necessity since absence or depletion of these cells could result in the complete depletion of one or more cell lineages, events that would lead within a short time to disease and death. Some of stem cells differentiate upon need, but some stem cells or their daughter cells produce other stem cells to maintain the precious pool of these cells. Thus, in addition to maintaining their own kind, pluripotential stem cells are capable of differentiation into several sublines of progenitor cells with more limited self-renewal capacity or no self-renewal capacity. These progenitor cells ultimately give rise to the morphologically recognizable precursor cells. The progenitor cells are capable of proliferating and differentiating along one, or more than one, of the myeloid differentiation pathways (Lajtha, L. G. (Rapporteur), 1979, Blood Cells 5:447).

Stem and progenitor cells make up a very small percentage of the nucleated cells in the bone marrow, spleen, and blood. About ten times fewer of these cells are present in the spleen relative to the bone marrow, with even less present in the adult blood. As an example, approximately one in one thousand nucleated bone marrow cells is a progenitor cell; stem cells occur at a lower frequency. These progenitor and stem cells have been detected and assayed for by placing dispersed suspensions of these cells into irradiated mice, and noting those cells that seeded to an organ such as the spleen and which found the environment conducive to proliferation and differentiation. These cells have also been quantified by immobilizing the cells outside of the body in culture plates (in vitro) in a semi-solid support medium such as agar, methylcellulose, or plasma clot in the presence of culture medium and certain defined biomolecules or cell populations which produce and release these molecules. Under the appropriate growth conditions, the stem or progenitor cells will go through a catenated sequence of proliferation and differentiation yielding mature end stage progeny, which thus allows the determination of the cell type giving rise to the colony. If the colony contains granulocytes, macrophages, erythrocytes, and megakaryocytes (the precursors to platelets, then the cell giving rise to them would have been a pluripotential cell. To determine if these cells have self-renewal capacities, or stemness, and can thus produce more of their own kind, cells from these colonies can be replated in vivo or in vitro. Those colonies, which upon replating into secondary culture plates, give rise to more colonies containing cells of multilineages, would have contained cells with some degree of stemness. The stem cell and progenitor cell compartments are themselves heterogeneous with varying degrees of self-renewal or proliferative capacities. A model of the stem cell compartment has been proposed based on the functional capacities of the cell (Hellman, S., et al., 1983, J. Clin. Oncol. 1:227–284). Self-renewal would appear to be greater in those stem cells with the shortest history of cell division, and this self-renewal would become progressively more limited with subsequent division of the cells.

A human hematopoietic colony-forming cell with the ability to generate progenitors for secondary colonies has been identified in human umbilical cord blood (Nakahata, T. and Ogawa, M., 1982, J. Clin. Invest. 70:1324–1328). In addition, hematopoietic stem cells have been demonstrated in human umbilical cord blood, by colony formation, to occur at a much higher level than that found in the adult (Prindull, G., et al., 1978, Acta Paediatr. Scand. 67:413–416; Knudtzon, S., 1974, Blood 43(3):357–361). The presence of circulating hematopoietic progenitor cells in human fetal blood (Linch, D. C., et al., 1982, Blood 59(5):976–979) and in cord blood (Fauser, A. A. and Messner, H. A., 1978, Blood 52(6):1243–1248) has also been shown. Human fetal and neonatal blood has been reported to contain megakaryocyte and burst erythroblast progenitors (Vainchenker, W., et al., 1979, Blood Cells 5:15–42), with increased numbers of erythroid progenitors in human cord blood or fetal liver relative to adult blood (Hassan, M. W., et al., 1979, Br. J. Haematol. 41:477–484; Tchernia, G., et al., 1981, J. Lab. Clin. Med. 97(3):322–331). Studies have suggested some differences between cord blood and bone marrow cells in the characteristics of CFU-GM (colony forming unit-granulocyte, macrophage) which express surface Ia antigens (Koizumi, S., et al., 1982, Blood 60(4):1046–1049).

U.S. Pat. No. 4,714,680 discloses cell suspensions comprising human stem and progenitor cells and methods for isolating such suspensions, and the use of the cell suspensions for hematopoietic reconstitution.

2.2. Reconstitution of the Hematopoietic System

Reconstitution of the hematopoietic system has been accomplished by bone marrow transplantation. Lorenz and coworkers showed that mice could be protected against lethal irradiation by intravenous infusion of bone marrow (Lorenz, E., et al., 1951, J. Natl. Cancer Inst. 12:197–201). Later research demonstrated that the protection resulted from colonization of recipient bone marrow by the infused cells (Lindsley, D. L., et al., 1955, Proc. Soc. Exp. Biol. Med. 90:512–515; Nowell, P. C., et al., 1956, Cancer Res. 16:258–261; Mitchison, N. A., 1956, Br. J. Exp. Pathol. 37:239–247; Thomas, E. D., et al., 1957, N. Engl. J. Med. 257:491–496). Thus, stem and progenitor cells in donated bone marrow can multiply and replace the blood cells responsible for protective immunity, tissue repair, clotting, and other functions of the blood. In a successful bone marrow transplantation, the blood, bone marrow, spleen,

thymus and other organs of immunity are repopulated with cells derived from the donor.

U.S. Pat No. 4,721,096 by Naughton et al. discloses a method of hematopoietic reconstitution which comprises obtaining and cryopreserving bone marrow, replicating the bone marrow cells in vitro, and then infusing the cells into a patient.

Bone marrow has been used with increasing success to treat various fatal or crippling diseases, including certain types of anemias such as aplastic anemia (Thomas, E. D., et al., Feb. 5, 1972, The Lancet, pp. 284–289), Fanconi's anemia (Gluckman, E., et al., 1980, Brit. J. Haematol. 45:557–564; Gluckman, E., et al., 1983, Brit. J. Haematol. 54:431–440; Gluckman, E., et al., 1984, Seminars in Hematology:21(1):20–26), immune deficiencies (Good, R. A., et al., 1985, Cellular Immunol. 82:36–54), cancers such as lymphomas or leukemias (Cahn, J. Y., et al., 1986, Brit. J. Haematol. 63:457–470; Blume, K. J. and Forman, S. J., 1982, J. Cell. Physiol. Supp. 1:99–102; Cheever, M. A., et al., 1982, N. Engl. J. Med. 307(8):479–481), carcinomas (Blijham, G., et al., 1981, Eur. J. Cancer 17(4):433–441), various solid tumors (Ekert, H., et al., 1982, Cancer 49:603–609; Spitzer, G., et al., 1980, Cancer 45:3075–3085), and genetic disorders of hematopoiesis. Bone marrow transplantation has also recently been applied to the treatment of inherited storage diseases (Hobbs, J. R., 1981, Lancet 2:735–739), thalassemia major (Thomas, E. D., et al., 1982, Lancet 2:227–229), sickle cell disease (Johnson, F. J., et al., 1984, N. Engl. J. Med. 311:780–783), and osteopetrosis (Coccia, P. F., et al., 1980, N. Engl. J. Med. 302:701–708) (for general discussions, see Storb, R. and Thomas, E. D., 1983, Immunol. Rev. 71:77–102; O'Reilly, R., et al., 1984, Sem. Hematol. 21(3):188–221; 1969, Bone-Marrow Conservation, Culture and Transplantation, Proceedings of a Panel, Moscow, Jul. 22–26, 1968, International Atomic Energy Agency, Vienna; McGlave, P. B., et al., 1985, in Recent Advances in Haematology, Hoffbrand, A. V., ed., Churchill Livingstone, London, pp. 171–197).

Present use of bone marrow transplantation is severely restricted, since it is extremely rare to have perfectly matched (genetically identical) donors, except in cases where an identical twin is available or where bone marrow cells of a patient in remission are stored in a viable frozen state. Even in such an autologous system, the danger due to undetectable contamination with malignant cells, and the necessity of having a patient healthy enough to undergo marrow procurement, present serious limitations. (For reviews of autologous bone marrow transplantation, see Herzig, R. H., 1983, in Bone Marrow Transplantation, Weiner, R. S., et al., eds., The Committee On Technical Workshops, American Association of Blood Banks, Arlington, Va.; Dicke, K. A., et al., 1984, Sem. Hematol. 21(2):109–122; Spitzer, G., et al., 1984, Cancer 54 (September 15 Suppl.):1216–1225). Except in such autologous cases, there is an inevitable genetic mismatch of some degree, which entails serious and sometimes lethal complications. These complications are two-fold. First, the patient is usually immunologically incapacited by drugs beforehand, in order to avoid immune rejection of the foreign bone marrow cells (host versus graft reaction). Second, when and if the donated bone marrow cells become established, they can attack the patient (graft versus host disease), who is recognized as foreign. Even with closely matched family donors, these complications of partial mismatching are the cause of substantial mortality and morbidity directly due to bone marrow transplantation from a genetically different individual.

Peripheral blood has also been investigated as a source of stem cells for hematopoietic reconstitution (Nothdurft, W., et al., 1977, Scand. J. Haematol. 19:470–481; Sarpel, S. C., et al., 1979, Exp. Hematol. 7:113–120; Ragharachar, A., et al., 1983, J. Cell. Biochem. Suppl. 7A:78; Juttner, C. A., et al., 1985, Brit. J. Haematol. 61:739–745; Abrams, R. A., et al., 1983, J. Cell. Biochem. Suppl. 7A:53; Prummer, O., et al., 1985, Exp. Hematol. 13:891–898). In some studies, promising results have been obtained for patients with various leukemias (Reiffers, J., et al., 1986, Exp. Hematol. 14:312–315 (using cryopreserved cells); Goldman, J. M., et al., 1980, Br. J. Haematol. 45:223–231; Ti ly, H., et al., Jul. 19, 1986, The Lancet, pp. 154–155; see also To, L. B. and Juttner, C. A., 1987, Brit. J. Haematol. 66: 285–288, and references cited therein); and with lymphoma (Korbling, M., et al., 1986, Blood 67:529–532). It has been implied that the ability of autologous peripheral adult blood to reconstitute the hematopoietic system, seen in some cancer patients, is associated with the far greater numbers of circulating progenitor cells in the peripheral blood produced after cytoreduction due to intensive chemotherapy and/or irradiation (the rebound phenomenon) (To, L. B. and Juttner, C. A., 1987, Annot., Brit. J. Haematol. 66:285–288; see also 1987, Brit. J. Haematol. 67:252–253, and references cited therein). Other studies using peripheral blood have failed to effect reconstitution (Hershko, C., et al., 1979, The Lancet 1:945–947; Ochs, H. D., et al., 1981, Pediatr. Res. 15(4 Part 2):601).

Studies have also investigated the use of fetal liver cell transplantation (Cain, G. R., et al., 1986, Transplantation 41(1):32–25; Ochs, H. D., et al., 1981, Pediatr. Res. 15(4 part 2):601; Paige, C. J., et al., 1981, J. Exp. Med. 153:154–165; Touraine, J. L., 1980, Excerpta Med. 514:277; Touraine, J. L., 1983, Birth Defects 19:139; see also Good, R. A., et al., 1983, Cellular Immunol. 82:44–45 and references cited therein) or neonatal spleen cell transplantation (Yunis, E. J., et al., 1974, Proc. Natl. Acad. Sci. U.S.A. 72:4100) as stem cell sources for hematopoietic reconstitution. Cells of neonatal thymus have also been transplanted in immune reconstitution experiments (Vickery, A. C., et al., 1983, J. Parasitol. 69(3):478–485; Hirokawa, K., et al., 1982, Clin. Immunol. Immunopathol. 22:297–304).

2.3. Cryopreservation of Cells

Freezing is destructive to most living cells. Upon cooling, as the external medium freezes, cells equilibrate by losing water, thus increasing intracellular,solute concentration. Below about 10–15° C., intracellular freezing will occur. Both intracellular freezing and solution effects are responsible for cell injury (Mazur, P., 1970, Science 168:939–949). It has been proposed that freezing destruction from extracellular ice is essentially a plasma membrane injury resulting from osmotic dehydration of the cell (Meryman, H. T., et al., 1977, Cryobiology 14:287–302).

Cryoprotective agents and optimal cooling rates can protect against cell injury. Cryoprotection by solute addition is thought to occur by two potential mechanisms: colligatively, by penetration into the cell, reducing the amount of ice formed; or kinetically, by decreasing the rate of water flow out of the cell in response to a decreased vapor pressure of external ice (Meryman, H. T., et al., 1977, Cryobiology 14:287–302). Different optimal cooling rates have been described for different cells. Various groups have looked at the effect of cooling velocity or cryopreservatives upon the survival or transplantation efficiency of frozen bone marrow cells or red blood cells (Lovelock, J. E. and Bishop, M. W. H., 1959, Nature 183:1394–1395; Ashwood-Smith, M. J.,

1961, Nature 190:1204–1205; Rowe, A. W. and Rinfret, A. P., 1962, Blood 20:636; Rowe, A. W. and Fellig, J., 1962, Fed. Proc. 21:157; Rowe, A. W., 1966, Cryobiology 3(1) :12–18; Lewis, J. P., et al., 1967, Transfusion 7(1):17–32; Rapatz, G., et al., 1968, Cryobiology 5(1):18–25; Mazur, P., 1970, Science 168:939–949; Mazur, P., 1977, Cryobiology 14:251–272; Rowe, A. W. and Lenny, L. L., 1983, Cryobiology 20:717; Stiff, P. J., et al., 1983, Cryobiology 20:17–24; Gorin, N. C., 1986, clinics in Haematology 15(1):19–48).

The successful recovery of human bone marrow cells after long-term storage in liquid nitrogen has been described (1983, American Type Culture Collection, Quarterly Newsletter 3(4):1). In addition, stem cells in bone marrow were shown capable of withstanding cryopreservation and thawing without significant cell death, as demonstrated by the ability to form equal numbers of mixed myeloid-erythroid colonies in vitro both before and after freezing (Fabian, I., et al., 1982, Exp. Hematol. 10(1):119–122). The cryopreservation and thawing of human fetal liver cells (Zuckerman, A. J., et al., 1968, J. Clin. Pathol. (London) 21(1):109–110), fetal myocardial cells (Robinson, D. M. and Simpson, J. F., 1971, In Vitro 6(5):378), neonatal rat heart cells (Alink, G. M., et al., 1976, Cryobiology 13:295–304), and fetal rat pancreases (Kemp, J. A., et al., 1978, Transplantation 26(4) :260–264) have also been reported.

2.4. Gene Therapy

Gene therapy refers to the transfer and stable insertion of new genetic information into cells for the therapeutic treatment of diseases or disorders. The foreign gene is transferred into a cell that proliferates to spread the new gene throughout the cell population. Thus stem cells, or pluripotent progenitor cells, are usually the target of gene transfer, since they are proliferative cells that produce various progeny lineages which will potentially express the foreign gene.

Most studies in gene therapy have focused on the use of hematopoietic stem cells. High efficiency gene transfer systems for hematopoietic progenitor cell transformation have been investigated for use (Morrow, J. F., 1976, Ann. N.Y. Acad. Sci. 265:13; Salzar, W., et al., 1981, in Organization and Expression of Globin Genes, A. R. Liss, Inc., New York, p. 313; Bernstein, A., 1985, in Genetic Engineering: Principles and Methods, Plenum Press, New York, p. 235; Dick, J. E., et al., 1986, Trends in Genetics 2:165). Reports on the development of viral vector systems indicate a higher efficiency of transformation than DNA-mediated gene transfer procedures (e.g., $CaPO_4$ precipitation and DEAE dextran) and show the capability of integrating transferred genes stably in a wide variety of cell types. Recombinant retrovirus vectors have been widely used experimentally to transduce hematopoietic stem and progenitor cells. Genes that have been successfully expressed in mice after transfer by retrovirus vectors include human hypoxanthine phosphoribosyl transferase (Miller, A., et al., 1984, Science 255:630). Bacterial genes have also been transferred into mammalian cells, in the form of bacterial drug resistance gene transfers in experimental models. The transformation of hematopoietic progenitor cells to drug resistance by eukaryotic virus vectors, has been accomplished with recombinant retrovirus-based vector systems (Hock, R. A. and Miller, A. D., 1986, Nature 320:275–277; Joyner, A., et al., 1983, Nature 305:556–558; Williams, D. A., et al., 1984, Nature 310:476–480; Dick, J. E., et al., 1985, Cell 42:71–79); Keller, G., et al., 1985, Nature 318:149–154; Eglitis, M., et al., 1985, Science 230:1395–1398). Recently, adeno-associated virus vectors have been used successfully to transduce mammalian cell lines to neomycin resistance (Hermonat, P. L. and Muzyczka, N., 1984, supra; Tratschin, J.-D., et al., 1985, Mol. Cell. Biol. 5:3251). Other viral vector systems that have been investigated for use in gene transfer include papovaviruses and vaccinia viruses (see Cline, M. J., 1985, Pharmac. Ther. 29:69–92).

Other methods of gene transfer include microinjection, electroporation, liposomes, chromosome transfer, and transfection techniques (Cline, M. J., 1985, supra). Salser et al. used a calcium-precipitation transfection technique to transfer a methotrexate-resistant dihydrofolate reductase (DHFR) or the herpes simplex virus thymidine kinase gene, and a human globin gene into murine hematopoietic stem cells. In vivo expression of the DHFR and thymidine kinase genes in stem cell progeny was demonstrated (Salser, W., et al., 1981, in Organization and Expression of Globin Genes, Alan R. Liss, Inc., New York, pp. 313–334).

Gene therapy has also been investigated in murine models with the goal of enzyme replacement therapy. Thus, normal stem cells from a donor mouse have been used to reconstitute the hematopoietic cell system of mice lacking beta-glucuronidase (Yatziv, S., et al., 1982, J. Lab. Clin. Med. 90:792–797). Since a native gene was being supplied, no recombinant stem cells (or gene transfer techniques) were necessary.

3. SUMMARY OF THE INVENTION

The present invention is directed to hematopoietic stem and progenitor cells of neonatal or fetal blood, that are cryopreserved, and the therapeutic uses of such stem and progenitor cells upon thawing. In particular, the present invention relates to the therapeutic use of fetal or neonatal stem cells for hematopoietic (or immune) reconstitution. Hematopoietic reconstitution with the cells of the invention can be valuable in the treatment or prevention of various diseases and disorders such as anemias, malignancies, autoimmune disorders, and other immune dysfunctions and deficiencies. In another embodiment, fetal or neonatal hematopoietic stem and progenitor cells which contain a heterologous gene sequence can be used for hematopoietic reconstitution in gene therapy.

In a preferred embodiment of the invention, neonatal or fetal blood cells that have been cryopreserved and thawed can be used for autologous (self) reconstitution.

The invention also relates to methods of collection and cryopreservation of the neonatal and fetal stem and progenitor cells of the invention.

3.1. Definitions

As used herein, the following abbreviations will have the meanings indicated:

ACD =	acid-citrate dextrose
BFU-E =	burst-forming unit-erythroid. An hematopoietic progenitor cell which is capable of producing a colony of erythroid progeny cells in semi-solid medium.
BFU-E-1 =	an early erythroid progenitor cell, capable of producing a colony of erythroid progeny cells in semi-solid medium upon stimulation by erythropoietin, hemin (optional), and a burst-promoting factor.

-continued

BFU-E-2 =	an erythroid progenitor cell, of greater maturity than BFU-E-1, which is capable of producing a colony of erythroid progeny cells in semi-solid medium upon stimulation by erythropoietin and by hemin (optional).
CFU =	colony-forming unit. A cell which is capable of producing a colony of progeny cells in semi-solid medium.
CFU-GEMM =	colony-forming unit-granulocyte, erythrocyte, monocyte/macrophage, megakaryocyte. A multipotential hematopoietic progenitor cell which is capable of producing a colony composed of granulocyte, erythrocyte, monocyte/macrophage, and megakaryocyte progeny, in semi-solid medium.
CFU-GM =	colony-forming unit-granulocyte, macrophage. An hematopoietic progenitor cell which is capable of producing a colony composed of granulocyte and macrophage progeny in semi-solid medium.
CFU-S =	colony forming unit-spleen. A multipotential stem cell with self-renewal capacity, which, upon inoculation into lethally-irradiated mice, is capable of producing a colony (nodules on the spleen(s).
CPD =	citrate-phosphate-dextrose
CSF =	colony stimulating factor
DMSO =	dimethyl sulfoxide
DNase =	deoxyribonuclease
DPBS =	phosphate buffered saline without magnesium or calcium
FCS =	fetal calf serum
heterologous gene =	a gene which is not present, or not functionally expressed, in the designated host cell.
IMDM =	Iscove's Modified Dulbecco's Medium
LD100/30 days =	the minimum or near-minimal Lethal Dosage causing 100% mortality within a 30-day post-irradiation period
PHALCM =	medium conditioned by phytohemagglutinin-stimulated leukocytes from patients with hemochromatosis
PWMSCM =	pokeweed mitogen spleen cell conditioned medium
S-cell =	stem cell
SLE =	systemic lupus erythematosus
^{3}HTdr =	tritiated thymidine
TLI =	total lymphoid irradiation

4. DESCRIPTION OF THE FIGURES

FIG. 1 presents data for neonatal blood volumes obtained in one series of collections from individual births. The volume (ml) of blood collected is shown along the X-axis, with infant weight (kg) along the Y-axis. Open circles represent births by Caesarian section; closed circles represent vaginal births.

FIG. 2 presents the data from neonatal blood volumes obtained in a second series of collections from individual births. The volume (ml) of blood collected is shown along the X-axis, with the infant weight (kg) along the Y-axis. Closed circles represent vaginal births, with collection by gravity drainage from the umbilical cord. Open circles represent births by Caesarian section, with collection by gravity drainage from the umbilical cord. Closed triangles represent vaginal births, with collection from the delivered placenta. open triangles represent births by Caesarian section, with collection from the delivered placenta.

FIGS. 3A and 3B are diagrammatic representations of the composition of centrifuge tubes at different steps in a Ficoll-Hypaque density separation, as described in Section 6.3.1, which can be employed to obtain low density cells that are enriched in hematopoietic stem and progenitor cells. The cord blood cell suspension is layered on Ficoll-Hypaque before centrifugation (FIG. 3A). After centrifugation, the low density cells appear as a sharp band between the Ficoll-Hypaque and the phosphate-buffered saline (FIG. 3B).

FIG. 4 is a diagrammatic representation of the apparatus described in Section 6.4, which can be used for the cryopreservation of neonatal and fetal hematopoietic stem and progenitor cells. The cryovials containing the cell suspensions are placed in a freezing rack which is in turn placed in a 4° C. methanol bath. The methanol bath (in a metal or glass freezing dish) is in turn placed in a −80° C. freezer. After the cells reach the frozen state, they are transferred to a long-term storage vessel containing liquid nitrogen.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to hematopoietic stem and progenitor cells of neonatal or fetal blood, that are cryopreserved, and the therapeutic uses of such stem and progenitor cells upon thawing.

In particular, the present invention relates to the use of fetal or neonatal stem cells for hematopoietic reconstitution. In a preferred embodiment of the invention, the fetal or neonatal stem cells can be used in autologous hematopoietic reconstitution, i.e., in the reconstitution of the hematopoietic system of the same individual from which they were originally derived. In such an embodiment, the invention provides substantial advantages over the present use of bone marrow for hematopoietic reconstitution. Present use of bone marrow transplantation is severely restricted by the fact that there is virtually never a perfectly matched (genetically identical) donor, except in cases where an identical twin is available or where bone marrow cells of, for example, a cancer patient in remission are stored in the viable frozen state in the hope that they will be free of malignant cells and healthy enough to be returned to the patient for treatment of any future relapse. Except in such cases, the inevitable genetic mismatch which results can entail the serious and sometimes lethal complications of host versus graft or graft versus host disease. In order to avoid host rejection of the foreign bone marrow cells (host versus graft reaction), the patient must be immunologically incapacitated. Such immune incapacitation is itself a cause of serious complications. Furthermore, when and if the donated bone marrow cells become established, they can attack the patient (graft versus host disease), who is recognized as foreign. Even with closely matched family donors, these complications of partial mismatching are the cause of substantial mortality and morbidity directly due to bone marrow transplantation from a genetically different individual.

In an embodiment of the invention directed to the use of neonatal stem and progenitor cells for hematopoietic reconstitution, there are several main reasons for preferring the use of such neonatal cells to conventional bone marrow transplantation. First, no donor is required because the cells can be obtained from neonatal blood that would otherwise be discarded. Second, in a preferred autologous system, i.e., involving use of "self" neonatal cells, the complications arising in conventional bone marrow transplantation from the need for pretransplantation drug-induced or irradiation-induced immune incapacitation and from acute and chronic graft-versus-host disease are all eliminated because, in this embodiment, neonatal cells are returned to their original

owner and are therefore totally compatible. For these reasons, present restrictions on the use of bone marrow transplantation arising from difficulties in finding even approximately matched donors, and from disease and mortality due to unavoidable genetic incompatibility, do not apply to self-reconstitution with neonatal cells. Third, regarding the preferred autologous embodiment, the efficiency of genetically identical (self) cells in bone marrow transplantation in animals is numerically many times greater than that of cells from a genetically dissimilar donor (Balner, H., 1977, Bone Marrow Transplantation and Other Treatment after Radiation Injury, Martinus Nijhoff Medical Division, The Hague), thus far fewer self cells are required for successful reconstitution in the preferred autologous system.

Furthermore, the prospects of success in bone marrow transplantation decline with age; although it is not clear whether the age of donor or patient is more important, it is proper to infer that younger (neonatal) cells are preferable for hematopoietic reconstitution. Such neonatal or fetal cells have not been subjected to the "environmental outrage" that adult cells have undergone. Also, as an example of novel medical applications which may be feasible with neonatal cells but not with conventional bone marrow transplantation, restoration with self cells taken at birth can be valuable in the treatment of disorders such as declining immune responsiveness and autoimmunity (immune reactions against one's own tissues) which occur in increasing frequency with age.

Many of the relative disadvantages discussed supra of the use of bone marrow cells for hematopoietic reconstitution, also apply to the use of adult peripheral blood for such reconstitution, and thus, the use of neonatal cells for hematopoietic reconstitution according to the present invention provides distinct advantages over the employment of adult peripheral blood. It has been implied that the ability of autologous peripheral adult blood to reconstitute the hematopoietic system, seen in some cancer patients, is associated with the Car greater numbers of circulating progenitor cells in the peripheral blood produced after cytoreduction due to intensive chemotherapy and/or irradiation (the rebound phenomenon) (To, L. B. and Juttner, C. A., 1987, Annot., Brit. J., Haematol. 66:285–288; see also 1987, Brit. J. Haematol. 67:252–253, and references cited therein). There are possible detrimental effects, known or unknown, of prior chemotherapy or irradiation, on the stem and progenitor cell populations found in these patients.

There are additional reasons for preferring the use of neonatal cells for hematopoietic reconstitution as provided by the present invention. Neonatal blood is a preferred source of cells for hematopoietic reconstitution, since it is free from viral and microbial agents, known or unknown, latent or otherwise, that may be encountered in later life, other than those transmitted from the mother or during labor and delivery. In addition, in view of the extent to which the hematopoietic stem cell may possibly share with other cells the limitation in total number of cell divisions that it may undergo before senescence, it is proper to assume that the neonatal hematopoietic stem cell has a self-renewal and reconstituting capacity that is at least as great, and perhaps greater, than that of hematopoietic stem cells obtained at any later time in life.

In adults, stem and progenitor cells are mostly confined to the bone marrow; very few circulate in the blood. In the newborn human or animal, however, stem and progenitor cells circulate in the blood in numbers similar to those found in adult bone marrow. Doubtless this reflects the great demands for blood formation of the growing infant. We calculate that the restorative capacity of neonatal blood contained in the human umbilical cord and placenta, which are customarily discarded at birth, equals or exceeds that of the average donation of an adult's bone marrow. The efficacy of human neonatal blood cells compared with adult bone marrow cells is gauged by laboratory assays for stem cell and progenitor cells. Progenitor cell assays imply that the reconstituting potential of cells from 50 ml of cord blood (readily obtainable) is at least equivalent to the average number of progenitor cells from adult bone marrow that is used in autologous hematopoietic reconstitution (see Section 6.8, infra). 'S-cells', representing probably the earliest developmental form of the stem cell, are demonstrable in human (cord) blood (Nakahata, T. and Ogawa, M., 1982, J. Clin. Invest. 70:1324–1328). Thus, the cells of neonatal blood can be judged an effective clinical substitute for adult bone marrow.

In laboratory animals, the efficacy of neonatal cells can be tested directly. Accordingly we have shown that circulating neonatal cells, in numbers lower than are contained in the cord and placenta, will completely and permanently repopulate the entire blood-forming and immune systems of a lethally irradiated adult animal, promoting complete recovery and return to normal health (see Section 6.11, infra).

The method of the invention may be divided into the following stages solely for the purpose of description: (a) isolation of fetal or neonatal hematopoietic stem and progenitor cells; (b) inspection and testing of fetal or neonatal blood; (c) enrichment for hematopoietic stem and progenitor cells; (d) cryopreservation; (e) recovery of stem and progenitor cells from the frozen state; (f) examination of cells recovered for clinical therapy; and (g) therapeutic uses in reconstitution of the hematopoietic system.

Since both fetal and neonatal hematopoietic cells are envisioned for use in the present invention, descriptions and embodiments of the invention herein described for neonatal cells are meant to apply equally to fetal cells, unless clearly otherwise indicated or apparent.

5.1. Isolation of Fetal or Neonatal Hematopoietic Stem and Progenitor Cells

Fetal or neonatal blood are sources of the hematopoietic stem and progenitor cells of the present invention.

Fetal blood can be obtained by any method known in the art. For example, fetal blood can be taken from the fetal circulation at the placental root with the use of a needle guided by ultrasound (Daffos, F., et al., 1985, Am. J. Obstet. Gynecol. 153:655–660; Daffos, F., et al., 1983, Am. J. Obstet. Gynecol. 146:985), by placentocentesis (Valenti, C., 1973, Am. J. Obstet. Gynecol. 115:851; Cao, A., et al., 1982, J. Med. Genet. 19:81), by fetoscopy (Rodeck, C. H., 1984, in Prenatal Diagnosis, Rodeck, C. H. and Nicolaides, K. H., eds., Royal College of Obstetricians and Gynaecologists, London), etc.

In a preferred embodiment of the invention, neonatal hematopoietic stem and progenitor cells can be obtained from umbilical cord blood and/or placental blood. The use of cord or placental blood as a source of cells to repopulate the hematopoietic system provides numerous advantages. Cord blood can be obtained easily and without trauma to the donor. In contrast, at present, the collection of bone marrow cells for transplantation is a traumatic experience which is costly in terms of time and money spent for hospitalization. Cord blood cells can be used for autologous transplantation, when and if needed, and the usual hematological and immunological problems associated with the use of allogeneic

cells, matched only partially at the major histocompatibility complex or matched fully at the major, but only partially at the minor complexes, are alleviated.

Collections should be made under sterile conditions. Immediately upon collection, the neonatal or fetal blood should be mixed with an anticoagulent. Such an anticoagulent can be any known in the art, including but not limited to CPD (citrate-phosphate-dextrose), ACD (acid citrate-dextrose), Alsever's solution (Alsever, J. B. and Ainslie, R. B., 1941, N. Y. St. J. Med. 41:126), De Gowin's Solution (De Gowin, E. L., et al., 1940, J. Am. Med. Ass. 114:850), Edglugate-Mg (Smith, W. W., et al.,. 1959, J. Thorac. Cardiovasc. Surg. 38:573), Rous-Turner Solution (Rous, P. and Turner, J. R., 1916, J. Exp. Med. 23:219), other glucose mixtures, heparin, ethyl biscoumacetate, etc. (See Hurn, B. A. L., 1968, Storage of Blood, Academic Press, New York, pp. 26–160). In a preferred embodiment, ACD can be used.

5.1.1. Collection of Neonatal Blood

The object of this aspect of the invention is to obtain a neonatal blood collection of adequate volume that is free of contamination. Since umbilical cord blood is a rich source of stem and progenitor cells (see Section 6.6, infra; Nakahata, T. and Ogawa, M., 1982, J. Clin. Invest. 70:1324–1328; Prindull, G., et al., 1978, Acta. Paediatr. Scand. 67:413–416; Tchernia, G., et al., 1981, J. Lab. Clin. Med. 97(3):322–331), the preferred source for neonatal blood is the umbilical cord and placenta. The neonatal blood can preferably be obtained by direct drainage from the cord and/or by needle aspiration from the delivered placenta at the root and at distended veins.

5.1.1.1. Volume

In a preferred embodiment, volumes of 50 ml or more of neonatal blood are obtained (see Section 6.1, infra).

Practical experience indicates that volumes of 50 ml or more are easily collected without additional measures in 80% of term births, and that collections of more than 40 ml are obtainable more than 90% of the time. Lower volumes may also be acceptable, and indicated under some circumstances (see Sections 5.1.1.2.3.1 and 5.1.1.2.3.2, infra).

The following information suggests that as little as 50 ml of cord blood contains enough of the appropriate cells to repopulate the hematopoietic system of an adult, and it is possible that even less cord blood would have the same effect:

1. In a small sampling of cases for autologous marrow transplantation (Spitzer, G., et al., 1980, Blood 55:317–323), rapid repopulation of hematopoiesis in patients with acute leukemia was associated with as few as 0.24 million granulocyte-macrophage progenitor cells (CFU-GM).

2. In human cord blood, there are approximately 50–200 CFU-GM per 100,000 low-density cells and at least 5 million low density cord blood cells per milliliter. Thus 50 milliliters of cord blood would contain in the range of 0.1 to greater than 0.5 million CFU-GM (see also Section 6.8, infra). The upper value agrees closely with estimations from the number of CFU-GM in 12.5 to 19 day old fetal blood (Lynch, D. C., et al., 1982, Blood 59:976–979).

3. Importantly, stem and progenitor cells in cord blood appear to have a greater proliferative capacity in culture dishes than those in adult bone marrow (Salahuddin, S. Z., et al., 1981, Blood 58:931–938; Cappellini, M. D., et al., 1984, Brit. J. Haematol. 57:61–70).

Significant to the use of cord blood as a source of stem cells, is that the assay for S-cells has been adapted for the growth of human cord blood (Nakahata, T. and Ogawa, M., 1982, J. Clin. Invest. 70:324–1328). All the known progenitor cells are present in cord blood in high numbers and this includes those progenitors for multilineages, granulocytes, macrophages, erythrocytes, mast cells, and basophils (id.; Fauser, A. A. and Messner, H. A., 1978, Blood 52:1243–1248; Koizumi, S., et al., 1982, Blood 60:1046–1049; Prindull, G., et al., 1978, Acta Paediatr. Scand. 67:413–416).

Furthermore, hematopoietic stem and progenitor cells can potentially be multiplied in culture, before or after cryopreservation, (see Sections 5.1.3.2, 5.3.2, infra), thus expanding the number of stem cells available for therapy.

5.1.1.2. Preferred Aspects

The following subsections provide detailed descriptions of preferred particular embodiments of the invention, and are intended for descriptive purposes only, in no way limiting the scope of the invention.

5.1.1.2.1. Collection Kit

In a preferred aspect, a collection kit, packaged in a sterile container, can be used. In one particular embodiment, the collection kit can consist of:

(i) a wide-mouth, graduated, collection container, with anticoagulant, into which the cut end of the cord may be placed for collection by gravity drainage. A small funnel can be provided for use if needed.

(ii) (optional) a plastic, flexible, sealed collection bag, similar to a donation bag, which has ports for injection of the collected blood, and contains anticoagulant.

(iii) an identification label, which identifies the infant source of the sample and time of collection.

For multiple births, separate collections, each performed with a separate kit, are preferred.

Sterilization of the containers can occur by any technique known in the art, including but not limited to beta-irradiation, autoclaving of suitable materials in a steam sterilizer, etc. For example, in a preferred embodiment, sterilization by beta-irradiation can be carried out by exposure to 2.5 megarads from a tungsten source (see Section 6.1, infra).

The collection kit may be placed in the surgical field in advance of a delivery, to afford ready availability.

5.1.1.2.2. Vaginal Delivery of the Term Infant

Vaginal delivery of the normal infant at term, spontaneously, by forceps, or as a breech delivery, should allow an ample collection of cord blood. After clamping the cord, the volume of fetal blood remaining in the cord and attached placenta has been estimated at 45 ml/kg infant body weight, or approximately 145 ml for a 7 lb (3.2 kg) baby (Hellman, L. M., et al., 1971, Williams Obstetrics, 14th Ed., Appleton-Century-Crofts, New York, p. 216).

Following delivery of the infant, by any method, with or without anesthesia, the infant is held in the plane of the vagina, and the cord is doubly cross-clamped and cut approximately three inches (7–8 cm) from the umbilicus. The infant is removed.

Maintaining usual sterile precautions, the cord is then transected just above the crushed portion in the clamp, and the resulting flow of fetal blood from umbilical vessels is caught in the container provided. An adequate collection can usually be accomplished without milking the cord, and is complete in approximately two minutes, before placental separation has occurred. Care should be taken to avoid contamination by maternal blood, urine, or other fluids in the delivery field. Blood in the container is then transferred to the bag provided for transport to the storage facility or, alternatively, the original container, if equipped with a tight screw cap, can itself be sent to the storage facility without transfer of its contents.

If, following infant delivery, events make collection at that time undesirable, collection can be done after delivery of the placenta (see Section 5.1.1.2.3.6, infra). If maternal infection is suspected, such a placental collection may be preferable. Collection can also be carried out by aspiration from the delivered placenta, in addition to gravity drainage.

In a most preferred embodiment, immediate cord clamping after delivery is carried out, in order to achieve collection of the greatest possible volume of cord blood. Studies have shown that the relative distribution of blood between the infant and placental circuits gradually shifts to the infant's blood circuits with increasing delay in cord clamping after delivery (Yao, A. C., et al., Oct. 25, 1969, Lancet: 871–873).

5.1.1.2.3. Other Circumstances of Birth and Delivery

5.1.1.2.3.1. Premature Birth

The cord blood of premature infants may contain an even greater proportion of stem and progenitor cells than full-term cord blood. Consequently, smaller volumes of cord blood from premature infant delivery may give as good a yield of stem and progenitor cells. (The use of stem and progenitor cell assays as described in Sections 5.4.2 and 6.6 can determine the yield). Thus, in general, cord blood collection should be carried out if premature infant survival is anticipated, even though the volume of blood collected may be less than usual. Collection procedures should be the same as for term births.

5.1.1.2.3.2. Multiple Births

Cord blood collections undertaken at the time of multiple births involve additional procedural considerations:

(i) Multiple births are often premature, and volumes of cord blood will be correspondingly smaller. Collections should be made nevertheless, so that the decision o preserve for storage can be made later.

(ii) When births of two or more infants occur, where use of the cord collection is envisioned for later self-reconstitution, it is essential that each cord collection be identified with the proper infant. In cases of doubtful zygosity, blood typing can be done on cord blood an-d postnatal samples.

(iii) The timing of twin cord blood collection can be at the discretion of the obstetrician (after delivery of one twin; or after delivery of both).

(iv) A careful description of the placental relationships should be made (single or double amnions; single, double or fused chorions).

5.1.1.2.3.3. Caesarian Delivery

Cord blood collections at caesarean section can be carried out with the same kit, and with the same procedure, as vaginal delivery. The cut end of the cord is lowered to promote gravity drainage.

At caesarean section, it is strongly preferred that the cord blood collection be made after delivery of the infant, and before placental separation. However, this may not be desirable in some instances, such as where there is brisk hemorrhage, the need to incise or separate an anteriorly implanted placenta, or preoccupation of personnel with other events in the operating field. Thus, in these and similar cases, the placenta can be removed, and cord blood collected from it later.

5.1.1.2.3.4. Complication Delivery

Complications of delivery arising from the condition of the mother or the infant, or both, may require the immediate and urgent attention of the obstetrician and his assistants. Under these circumstances, the delivered placenta can be placed to one side, and collection carried out as soon as feasible.

5.1.1.2.3.5. Abnormal Placenta

For successful cord blood collection, it is preferred that the placenta be intact, or nearly so. Cases of marginal or partial separation can still offer an opportunity for collection, although it may have to be carried out after delivery of the placenta, if clinical circumstances indicate a need for prompt removal. Collections will be disfavored for use if a rupture of fetal circulation has occurred. Samples can be tested later for contamination by maternal blood (see Section 5.1.2, infra). Accurate description of the placental abnormality is preferred.

5.1.1.2.3.6. Collection from the Delivered Placenta

When rapid delivery of the placenta occurs or becomes necessary, and cord blood collection cannot be accomplished prior to placental separation, a sample of sufficient volume can still be obtained after delivery. The placenta and attached cord, still clamped, are placed to one side, but still within the sterile field. Collection is by the same technique described supra in section 5.1.1.2.2. It is preferred, however, that collection be completed within five minutes of delivery, while maintaining sterile procedures.

Cord blood collection prior to placental separation is preferred over collection from the delivered placenta for the following reasons: In a collection from delivered placenta, (i) collection volumes are generally less; (ii) some degree of clotting in the placental circulation may restrict recovery, and (iii) the likelihood of contamination, by maternal blood or other agents, is increased. Therefore, the determination of suitability of the sample collected from a delivered placenta is especially important.

5.1.1.2.3.7. Medical Conditions of the Mother

Given the general prohibition against maternal use of drugs which would adversely affect the fetus, it is unlikely that maternal therapy or medical status in the general sense would adversely affect stem cell retrieval from cord blood collection of a normal infant. In a preferred embodiment, however, specific information should be obtained in regard to drug abuse, vira-diseases capable of vertical transmission, and the influence of acute maternal illness at the time of delivery, since it is possible that these may affect stem cell retrieval from cord blood.

5.1.1.2.3.8. Unplanned Delivery

Despite elaborate plans, delivery may occur inopportunely, sometimes prematurely, and without the immediate services of a physician. Under these circumstances, the following procedures are preferred: (i) cord blood collection should be attempted with the standard kit, described supra; (ii) the placenta, if delivered on an unsterile field, should simply be kept as clean as possible, left with the cord clamped, and collection attempted within 5 minutes; (iii) the cord should be wiped with a cleansing agent (e.g. Betadine), and transected above the clamp, to make the collection; and (iv) circumstances of the delivery should be described with the specimen.

5.1.1.2.4. Recordation of Data

In a preferred embodiment, the data listed in Table I, infra, are obtained at the time of collection in order to ensure the accurate identification and evaluation of the collected blood.

TABLE I

DATA TO BE RECORDED AT THE TIME OF NEONATAL BLOOD COLLECTION
Date and time of delivery
Full name and address of mother
Hospital identification

TABLE I-continued

DATA TO BE RECORDED AT THE TIME OF NEONATAL BLOOD COLLECTION
Sex of infant
Weight of infant
Birth order (for multiple pregnancies)
Gestational age
Pregnancy complications
Intrapartum complications
Type of delivery
Placental collection (amount of blood collected)
Placental description and weight
Condition of infant

5.1.2. Inspection and Testing of Neonatal Blood

In a preferred embodiment, the neonatal blood sample is inspected and tested to ensure its suitability. Appropriate inspections and tests include but are not limited to the procedures described infra.

If the blood collection sample is to be shipped to a processing plant, the blood container and its contents should be inspected for defects such as inadequate closure and leakage. As an option, the collection kit may include a suitably positioned reusable maximum-minimum mercury thermometer to register the range of temperature change during shipment. Clots, opacity of the plasma and visible hemolysis are indications of bacterial contamination or other consequences of faulty handling. Time elapsed since collection can be noted.

The following tests on the collected neonatal blood sample can be performed either routinely, or where clinically indicated:

(i) Bacterial culture: To ensure the absence of microbial contamination, established assays can be performed, such as routine hospital cultures for bacteria under aerobic and anaerobic conditions.

(ii) Diagnostic screening for pathogenic microorganisms: To ensure the absence of specific pathogenic microorganisms, various diagnostic tests can be employed. Diagnostic screening for any of the numerous pathogens transmissible through blood can be done by standard procedures. As one example, the collected blood sample can be subjected to diagnostic screening for the presence of Human Immunodeficiency Virus (HIV), the causative agent of Acquired Immune Deficiency Syndrome (AIDS) (Gallo et al., 1984, Science 224:500–503; Barre-Sinoussi, F., et al., 1983, Science 220:868; Levy, J. A., et al., 1984, Science 225:840). Any of numerous assay systems can be used, based on the detection of virions, viral-encoded proteins, HIV-specific nucleic acids, antibodies to HIV proteins, etc.

(iii) Confirmation of neonatal origin of the blood: Contamination with maternal blood, not necessarily a contraindication to storage and clinical utility, may be suspected from the obstetrical history. Presence of maternal cells, and of adult blood generally, can be revealed by various tests, including but not limited to I typing (Wiener, A. S., et al., 1965, Am. J. Phys. Anthropol. 23(4): 389–396); analysis on a Coulter Channelyzer, which detects size differences between neonatal and maternal blood cells (Daffos, F., et al., 1985, Am. J. Obstet. Gynecol. 153:655–660); staining procedures for hemoglobin such as the Kleinhauer-Betke technique (Betke, K., 1968, Bibl. Haematologica 29:1085) and others (Clayton, E. M., et al., 1970, Obstetrics and Gynecology 35(4):642–645), which detect differences in the types of hemoglobin contained in red blood cells before birth versus in later life; etc.

In a preferred embodiment, I typing can be done by established methods, such as agglutination with anti-i and anti-I antibodies. Erythrocytes of neonates are i strong, c weak; by 18 months of age, erythrocytes are I strong; i weak (Marsh, W. L., 1961, Brit. J. Haemat. 7:200). Thus, the degree of reaction with anti-i or anti-I antibodies is a measure of the proportion of neonatal blood and red cells in a mixture of neonatal and adult blood. The corresponding contamination with maternal stem and progenitor cells would be far less than the total maternal cell contamination since the stem and progenitor cells are rare in adult blood. (Scarcity of stem and progenitor cells in colony assays (see Sections 5.4.2 and 6.6, infra) is another distinction between neo natal and adult blood.)

5.1.3. Optional Procedures

In a preferred embodiment of the invention, whole neonatal blood, as collected, can be cryogenically frozen, thus minimizing cell losses which can be incurred during cell processing protocols. However, cell separation procedures and expansion of stem and progenitor cells in in vitro cultures remain options. Such procedures may be useful, e.g., in reducing the volume of sample to be frozen, and increasing cell count, respectively. The procedures described infra in Sections 5.1.3.1 and 5.1.3.2 should be carefully screened before use, in order to ensure that hematopoietic stem and progenitor cell loss in processing does not endanger the therapeutic efficacy of a collected blood sample in hematopoietic reconstitution.

5.1.3.1. Enrichment for Hematopoietic Stem and Progenitor Cells: Cell Separation Procedures

After receiving cord blood or bone marrow samples in anticoagulant (e.g., ACD), the cells can be subjected to physical and/or immunological cell separation procedures. Such procedures enrich for the hematopoietic stem and progenitor cells so that fewer total cells have to be stored and transplanted. However, if cell separation is desired, care should be taken to ensure sufficient recovery of the hematopoietic stem and progenitor cells.

Various procedures are known in the art and can be used to enrich for the stem and progenitor cells of the present invention. These include but are not limited to equilibrium density centrifugation, velocity sedimentation at unit gravity, immune rosetting and immune adherence, counterflow centrifugal elutriation, T lymphocyte depletion, and fluorescence-activated cell sorting, alone or in combination. Recently, procedures have been reported for the isolation of very highly enriched populations of stem/progenitor cells. Murine CFU-S have been purified by several groups using slightly different procedures (Visser, J. W. M., et al., 1984, J. Exp. Med. 59:1576; Nijhof, W., et al., 1984, Exp. Cell Res. 155:583; Bauman, J. G. J., et al., 1986, J. Cell. Physiol. 128:133; Lord, B. I. and Spooncer, E., 1986, Lymphokine Res. 5:59). Studies using human (Emerson, S. G., et al., 1985, J. Clin. Invest. 76:1286) or murine (Nicola, N. A., et al., 1981, Blood 58:376) fetal liver cells have yielded highly enriched progenitor cells with up to 90% of them being colony forming cells for multi-, erythroid-, and granulocyte-macrophage lineages. CFU-E have also been very highly enriched (Nijhof, W., et al., 1983, J. Cell Biol. 96:386). Purification of adult mouse marrow CFU-GM with cloning efficiencies of up to 99% in semi-solid medium has been accomplished by pretreatment of mice three days prior to sacrifice with cyclophosphamide, density separation of cells on Ficoll-Hypaque, and counterflow centrifugal elutriation (Williams, D. E., et al., 1987, Exp. Hematol. 15:243). The